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CONTENTS

No. 1. February, 1935

PAGE

Parker, G. H.
The Electric Stimulation of the Chromatophoral Nerve-Fibers in the Dogfish 1

Parker, G. H., and Helen Porter Brower
A Nuptial Secondary Sex Character in Fundulus heteroclitus .. 4

Sears, Mary
Responses of Deep-seated Melanophores in Fishes and Amphibians 7

Torvik-Greb, Magnhild
The Chromosomes of Habrobracon 25

Shull, A. Franklin
Combinations of Current and Antecedent Conditions in Relation to Wing-production of Aphids 35

Kidder, George W., and Francis M. Summers
Taxonomic and Cytological Studies on the Ciliates Associated with the Amphipod Family Orchestiidae from the Woods Hole District. I. The stomatomous holotrichous ectocommensals .. 51

Chao, Iping
Hydrogen-ion Concentration and the Rhythmic Activity of the Nerve Cells in the Ganglion of the Limulus Heart 69

Rugh, Roberts
Pituitary-Induced Sexual Reactions in the Anura 74

Elliott, Alfred M.
The Influence of Pantothenic Acid on Growth of Protozoa 82

ZoBell, Claude E., and George F. McEwen
The Lethal Action of Sunlight upon Bacteria in Sea Water .. 93

Berkeley, C.
The Chemical Composition of the Crystalline Style and of the Gastric Shield; with Some New Observations on the Occurrence of the Style Oxidase 107

Leavitt, Benjamin B.
A Quantitative Study of the Vertical Distribution of the Larger Zoöplankton in Deep Water 115

44543

SMITH, DIETRICH C., AND MARGARET T. SMITH
Observations on the Color Changes and Isolated Scale Erythrophores of the Squirrelfish, Holocentrus ascensionis (Osbeck) .. 131
SPEIDEL, CARL CASKEY
Studies of Living Nerves 140

No. 2. APRIL, 1935

PAGE

KLEINHOLZ, L. H.
The Golgi Bodies in the Nerve Cells of the Crayfish, Cambarus 163
NUTTYCOMBE, JOHN W., AND AUBREY J. WATERS
Stenostomum pseudoacetabulum (nov. spec.) 168
SCHECHTER, VICTOR
The Effect of Centrifuging on the Polarity of an Alga, Griffithsia bornetiana 172
GOLDFORB, A. J.
Change in Size and Shape of Ageing Eggs (Arbacia punctulata) 180
GOLDFORB, A. J.
Viscosity Changes in Ageing Unfertilized Eggs of Arbacia punctulata 191
HASLER, ARTHUR D.
The Physiology of Digestion of Plankton Crustacea. I. Some digestive enzymes of Daphnia 207
HETHERINGTON, DUNCAN C., AND MARY E. SHIPP
The Effect of Cupric, Manganous and Ferric Chlorides upon Cardiac Explants in Tissue Culture 215
CLARKE, G. L., AND S. S. GELLIS
The Nutrition of Copepods in Relation to the Food-Cycle of the Sea 231
WELSH, JOHN H.
Further Evidence of a Diurnal Rhythm in the Movement of Pigment Cells in Eyes of Crustaceans 247
BURGER, J. WENDELL, AND CHARLES STEAD THORNTON
A Correlation between the Food Eggs of Fasciolaria tulipa and the Apyrene Spermatozoa of Prosobranch Molluscs 253
RANDALL, MERLE, AND THOMAS C. DOODY
A Buffered and Low Oxygen Content Physiological Salt Solution 258
WITSCHI, EMIL
The Chromosomes of Hermaphrodites. I. Lepas anatifera L. 263
MORGAN, T. H.
Centrifuging the Eggs of Ilyanassa in Reverse 268

MORGAN, T. H.
The Separation of the Egg of Ilyanassa into Two Parts by Centrifuging 280
MORGAN, T. H.
The Rhythmic Changes in Form of the Isolated Antipolar Lobe of Ilyanassa 296
BISSONNETTE, THOMAS HUME
Modification of Mammalian Sexual Cycles. II 300
OESTING, R. B., AND W. C. ALLEE
Further Analysis of the Protective Value of Biologically Conditioned Fresh Water for the Marine Turbellarian, Procerodes Wheatlandi. IV. The effect of calcium 314
RUNNSTRÖM, JOHN
On the Influence of Pyocyanine on the Respiration of the Sea Urchin Egg 327

NO. 3. JUNE, 1935

PAGE
DAWSON, ALDEN B.
The Hemopoietic Response in the Catfish, Ameiurus nebulosus, to Chronic Lead Poisoning 335
KORR, IRVIN M.
The Relation between Cell Integrity and Bacterial Luminescence 347
EVANS, LLEWELLYN THOMAS
The Effects of Antuitrin S and Sheep Pituitary Extract on the Female Lizard, Anolis carolinensis 355
MENKE, JOHN F.
The Hemolytic Action of Photofluorescein 360
SHAPIRO, HERBERT
The Validity of the Centrifuge Method for Estimating Aggregate Cell Volume in Suspensions of the Egg of the Sea-Urchin, Arbacia punctulata 363
RUNNSTRÖM, JOHN
An Analysis of the Action of Lithium on Sea Urchin Development 378
WISLOCKI, GEORGE B.
The Lungs of the Manatee (Trichechus latirostris) Compared with Those of Other Aquatic Mammals 385
JOHNSON, MARTIN W.
The Developmental Stages of Labidocera 397
DROUET, FRANCIS, AND AARON COHEN
The Morphology of Gonyostomum semen from Woods Hole, Massachusetts 422

LOEB, LEO
Comparison of the Reactions Against Heterotransplanted Tissues in Different Kinds of Hosts 440
TYLER, ALBERT
On the Energetics of Differentiation. II. A Comparison of the Rates of Development of Giant and of Normal Sea-urchin Embryos 451
VOLUME INDEX 461

Vol. LXVIII, No. 1 February, 1935

THE

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THE ELECTRIC STIMULATION OF THE CHROMATOPHORAL NERVE-FIBERS IN THE DOGFISH

G. H. PARKER

(From the Woods Hole Oceanographic Institution[1] *and the Marine Biological Laboratory, Woods Hole, Mass.)*

In the chromatophoral color changes of the common dogfish, *Mustelus canis,* whereby this fish may assume a dark or a light coloration, it has been shown that the dark phase is excited by secretions from the pituitary gland (Lundstrom and Bard, 1932) and that the light one is due to the action of concentrating nerve-fibers (Parker and Porter, 1934). Parker and Porter stimulated these fibers by cutting them transversely, a procedure that was particularly successful when it was carried out near the base of a fin. Under such circumstances a light band quickly appeared on the fin in what was obviously the area of peripheral distribution for the severed nerve-fibers. That the operation had caused no serious disturbance in the blood supply to the region concerned could be shown by a microscopic examination of the living skin in the band itself. By this means an unimpeded flow of blood in the light area could easily be demonstrated. Parker and Porter employed no other method for stimulating the chromatophoral nerve-fibers than cutting them. In the present paper it is intended to show that an electric current from an inductorium is as efficient a means of stimulating these fibers as is their severance.

It was found convenient to experiment on the anterior dorsal fin of the dogfish. This fish, with its dorsal fin uppermost, can be tied to a wooden carrier and kept indefinitely quiescent by providing it with a current of sea water over its gills. Near the root of its dorsal fin and not far from the anterior edge of this structure a cut 5 mm. long, transverse to the fin rays and completely through the fin was made as a means of exciting a peripheral light band. Posterior to this cut and in line with it two moderately large needle holes were made through the fin and about 5 mm. apart. Into each hole was inserted the platinum electrode from an electric inductorium which, however, was not yet in operation. The whole preparation was allowed to stand for half an hour. During this time a well-developed light band formed in the region of the fin peripheral to the cut but no change was noticeable in that part of the fin that was distal to the two holes. Having been assured that the making of the holes and the insertion into them of the two electrodes had not changed the coloration of the fin, I now started the inductorium. About ten minutes after this had been done a light area began to appear in the fin peripheral to the holes and in 25 minutes it was well pronounced and essentially indistinguishable from that which had resulted from the cutting of the fin (Fig. 1). This test was carried out on four fishes and with similar results in all cases.

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[1] Contribution No. 60.

When a fin into which electrodes have been inserted is simultaneously stimulated both by making the current and by cutting the nerve-fibers in positions such as have already been described, the formation of the two light bands can be watched step by step. Under such circumstances they will be seen to form at essentially the same rate and to the same degree. Thus there seems to be no real difference in the light bands formed by these two methods and I therefore conclude that stimulation by an electric current is as effective for the concentrating melanophore nerve-fibers as is cutting.

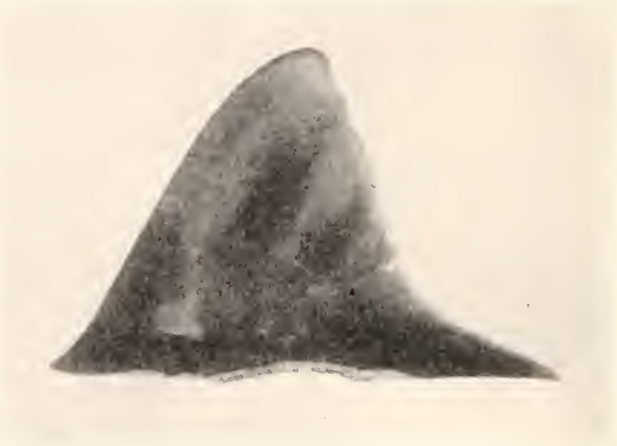

FIG. 1. Photographic side view of the anterior dorsal fin of a dark common dogfish, *Mustelus canis*, which has been stimulated to the formation of light bands by a transverse cut near the anterior edge of the fin and by electric stimulation posterior to this cut. The two holes into which the electrodes were inserted can be seen as dots in line with the transverse cut but posterior to it. The resultant light areas are seen in the fin peripheral to the two regions of stimulation.

In *Fundulus* the cutting of the chromatophoral nerve-fibers stimulates the dispersion of melanophore pigment, not its concentration. Electrical stimulation of these fibers, on the other hand, stimulates concentration, not dispersion (Mills, 1932). In my opinion these conditions are not the result of an exclusive action of the two means of stimulation on different classes of fibers. Both concentrating fibers and dispersing fibers are in all probability excited by each agent. The difference is due, I believe, to the greater ease with which concentrating fibers can be excited electrically and dispersing fibers by cutting. In the dogfish, where only one set of fibers is present, those concerned with pigment concentration, both types of stimulation are effective in bringing about the same reaction. These reactions are best seen in dogfishes that have not reached the stage of maximum darkness but which are only moderately dark. This is probably due to the great effectiveness of the pituitary secretion by which the dogfish melanophores are excited to disperse their pigment as contrasted with the weaker effect of the concentrating nerve-fibers which induce the opposite change (Parker and Porter, 1934).

Conclusion

The concentrating melanophore nerve-fibers of the dogfish can be excited to action whereby the fish may be locally blanched as well through *electric* stimulation as through *mechanical* stimulation (cutting).

REFERENCES

Lundstrom, H. M., and P. Bard, 1932. Hypophysial Control of Cutaneous Pigmentation in an Elasmobranch Fish. *Biol. Bull.*, **62**: 1.

Mills, S. M., 1932. The Double Innervation of Fish Melanophores. *Jour. Exper. Zool.*, **64**: 231.

Parker, G. H., and H. Porter, 1934. The Control of the Dermal Melanophores in Elasmobranch Fishes. *Biol. Bull.*, **66**: 30.

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A NUPTIAL SECONDARY SEX-CHARACTER IN FUNDULUS HETEROCLITUS

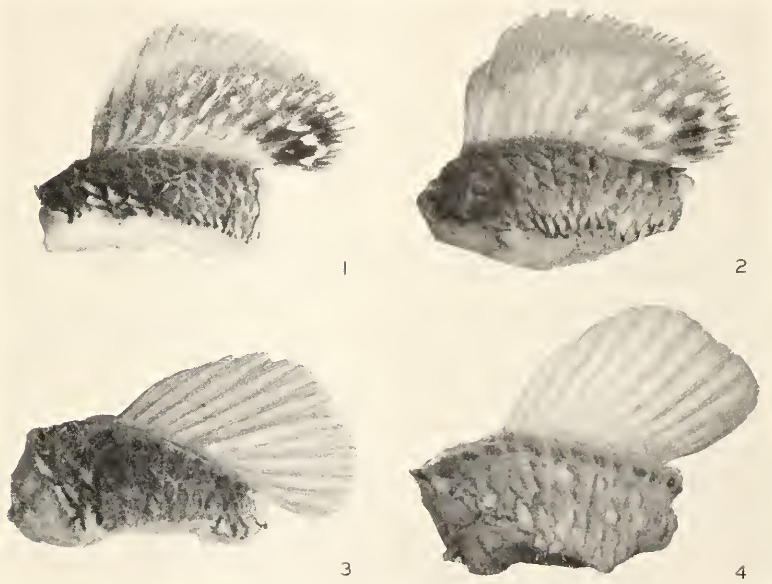
GEORGE HOWARD PARKER AND HELEN PORTER BROWER

(*From the Biological Laboratories, Harvard University*)

Everyone who has selected *Fundulus* for eggs or sperm is familiar with the striking coloration pattern in the males and especially with the dark splotch in the posterior part of the dorsal fin in this sex (Newman, 1907). This splotch is made up of melanophores and is not without interest. To test its constancy in relation to sex one hundred killifishes were opened during the breeding season (June, 1934) and their sex determined by an inspection of their gonads. Forty-seven of these fishes proved to be males and fifty-three females. All the males exhibited the fin-mark already alluded to and none of the females showed it. Apparently it is a character accurately associated with sex.

It is in all essential respects undisturbed by the change of *Fundulus* from the dark to the light condition or the reverse. Figure 1 shows the dorsal fin of a male fish in the dark phase, and notwithstanding the generally deep tone of the fin, the nuptial mark stands out conspicuously. The anterior three-fourths of the free edge of the fin contains so few melanophores as to appear quite light. The rest of the fin carries the nuptial mark composed of a great abundance of large melanophores. Here and there in this part of the fin are elongated light-spots parallel with the rays. The last few interrays near the posterior edge of the fin are, on the whole, densely black with several striking light-spots that add intensity to the mark. This darkened area and especially its posterior portion is what we have called the nuptial mark. In the light condition of the males the mark, especially its posterior part, is still conspicuous (Fig. 2). In the female the dorsal fin is uniformly dotted over with melanophores which neither in the dark state (Fig. 3) nor in the light one (Fig. 4) show any special concentration.

About a quarter of an hour after a male *Fundulus* has been put into a white-walled illuminated vessel it will have become extremely light-tinted in that the pigment in its skin melanophores will have assumed a high degree of concentration. The nuptial mark, however, will remain under these circumstances conspicuously dark as though the pigment of its melanophores had failed to concentrate. If, however, this spot is examined under the microscope it will be seen to be made up of many melanophores, both large and small, in all of which the pigment is strongly concentrated. Their number, however, is so great as compared with that in equal areas of other parts of the body that notwithstanding the state of their pigment they form a dark area. But the nuptial spot is not only a rich aggregation of melanophores; it is a region in which a large amount of free melanin occurs. This melanin is in the form of clumps in the tissue spaces of the skin and probably results from the disintegration of color-cells in which it was once contained. In this way the nuptial mark in a light fish remains a conspicuous feature even though the pigment in its melanophores may have gone into a concentrated state.



Four enlarged views of the dorsal fins of *Fundulus heteroclitus,* two from males and two from females, one in each sex for the light condition and the other for the dark. In each instance the anterior edge of the fin is to the left. Photographs by Dr. F. M. Carpenter.

Fig. 1. Dorsal fin of a male in dark condition.
Fig. 2. Dorsal fin of a male in light condition.
Fig. 3. Dorsal fin of a female in dark condition.
Fig. 4. Dorsal fin of a female in light condition.

Fundulus, some ten minutes after it has received a subcutaneous injection of adrenalin, will become extremely light in that the pigment in both its large and its small melanophores will have become maximally concentrated. But even in this extreme state the male nuptial mark is still conspicuous and for the same reason as that already given; its melanophores are so numerous that even though their pigment is concentrated by adrenalin, they nevertheless constitute a dark area in the fin of a light fish. Their response to adrenalin allies them with the melanophores of the rest of the fish, but puts them in contrast with the yellow and red cells in the so-called "Hochzeitskleid" of *Phoxinus*, which, according to Abolin (1925*a*) and to Giersberg (1930) are not influenced by adrenalin.

The male nuptial mark has an interesting history throughout the year. This history has been followed for some eighteen months on material in part from Woods Hole and in part from the neighborhood of Boston, Massachusetts. No nuptial marks have been noticed in male *Fundulus* from these localities between the months of November and of March. In April the mark begins to appear. It is present in May, is well pronounced in June and July, diminishes in August, and disappears in November. It is therefore quite clearly a mark of the breeding season, as in fact the generally high coloration of the male also is. It does not, however, belong to that group of color changes such as have been studied recently by Wunder (1931) in *Rhodeus* and which momentarily flash up in the male during the breeding act. It is a permanent sign of the whole breeding season and its limitation to one sex suggests that it may result from some male neurohumor produced during that period and acting on a specially receptive area in the dorsal fin of the fish. (See Abolin, 1925*b*.)

SUMMARY

The male of *Fundulus heteroclitus* possesses a secondary sex-character in the form of a melanophoric mark on its dorsal fin to be seen from April to November but not at other times of the year. It may therefore be described as nuptial. It does not occur in the female. It is composed of an unusually dense aggregation of melanophores whose melanin even when concentrated (light phase) forms a conspicuous dark mark.

REFERENCES

ABOLIN, L., 1925*a*. Beeinflussung des Fischfarbwechsels durch Chemikalien. I. Infundin- und Adrenalin-wirkung auf die melano- und Xanthophoren der Elritze (Phoxinus laevis Ag.). *Arch. mikr. Anat. Entw.-mech.*, **104**: 667.

ABOLIN, L., 1925*b*. Beeinflussung des Fischfarbwechsels durch Chemikalien. II. Annahme mannlicher Erythrophorenfärbung durch das infundinisierte Weibchen der Elritze (Phoxinus laevis Ag.). *Anz. Akad. Wiss. Wien., math.-nat. Kl.*, **61**: 172.

GIERSBERG, H., 1930. Der Farbwechsel der Fische. *Zeitschr. vergl. Physiol.*, **13**: 258.

NEWMAN, H. H., 1907. Spawning behavior and sexual dimorphism in Fundulus heteroclitus and allied fish. *Biol. Bull.*, **12**: 314.

WUNDER, W., 1931. Experimentelle Erzeugung des Hochzeitskleides beim Bitterling (Rhodeus amarus) durch Einspritzung von Hormonen. *Zeitschr. vergl. Physiol.*, **13**: 696.

RESPONSES OF DEEP-SEATED MELANOPHORES IN FISHES AND AMPHIBIANS

MARY SEARS

(From the Zoölogical Laboratories, Radcliffe College)

Introduction

Although there have been numerous investigations of the activities of the skin melanophores (Parker, 1930), only a few have included the deep-seated melanophores. These (Lieben, 1906; Ogneff, 1908; Hooker, 1912; Allen, 1917; Smith, 1916; Fischel, 1920; Uyeno, 1922; Gilson, 1926; Yamamoto, 1931) suggest little correlation in the activities of the two sets of melanophores. In amphibians, the absence of any marked response in the deep-seated cells to such drugs as adrenalin may have been due to a degenerate condition, since most of the earlier work was carried out after killing the animal. In fish, various reactions have been found, some identical to those in the skin, others the reverse. Because of these results, it seemed well to examine the activities of the deep-seated melanophores in animals in which the behavior of the dermal melanophores had been studied, by the use of the same agents which affect these cells in the skin, to learn whether the reactions of the deep-seated melanophores are comparable with those in the skin. The animals chosen were the common leopard frog, *Rana pipiens* Schreber, the tadpole of *Rana clamitans* Latreille, and the killifish, *Fundulus heteroclitus* L.

This work was carried out at the suggestion of Professor G. H. Parker to whom I am indebted for his constant supervision and advice.

Methods

The three species used, on a black background assume a dark coloration by expansion of their dermal melanophores, and on a white one, a light shade by contraction of these cells. Such conditions may also be induced by hormones, adrenalin producing a light appearance and pituitrin, at least in amphibians, a dark one. In extending these tests to the deep-seated melanophores, white porcelain dishes served for one extreme of background and battery jars painted externally with dull black, the other. In adrenalin tests, the animals were confined in the black jars, and in the pituitrin tests, they were placed in the white dishes, some time before injecting and kept there until they were killed. Throughout all experiments the animals were illuminated from a western window or by an electric lamp.

As the condition of the deep-seated melanophores may be inspected only upon killing the individual, a group of animals was subjected to the various tests and the internal melanophores of a number of these were examined at intervals during such trials to trace the activities of these cells step by step. Caution was taken that no rearrangement in the position of the pigment within the internal cells occurred during dissection. In frogs and tadpoles, whose dermal melanophores are chiefly under humoral control, no perceptible change appeared after considerable handling or for some hours after pithing. Hence, the state of the internal melanophores within a minute or two of pithing seemed indicative of the living condition. However, in killifish, whose melanophores are almost entirely under nervous control, these cells expanded at once

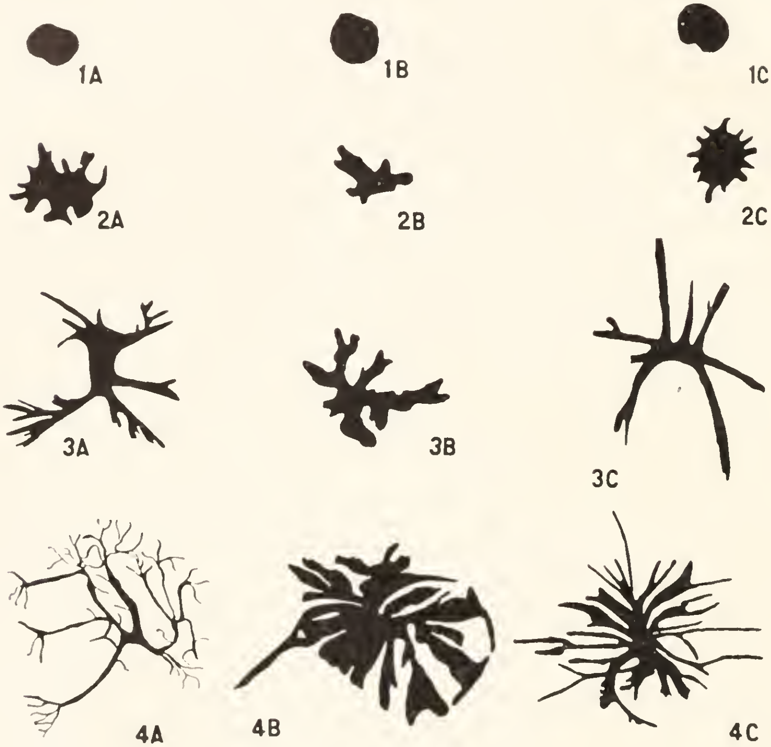


PLATE I

Semidiagrammatic drawings of dermal melanophores from the frog (*A*), the tadpole (*B*), and the killifish (*C*) showing four stages of expansion: 1, punctate condition; 2, stellate condition; 3, incomplete expansion; 4, complete expansion.

on manipulation and especially on sectioning their nervous connections. To overcome this, the deep-seated tissues were examined without first destroying the brain, by cutting away the body wall on one side without severing the nerves to the parietal peritoneum of the opposite wall or to the visceral peritoneum and mesenteries.

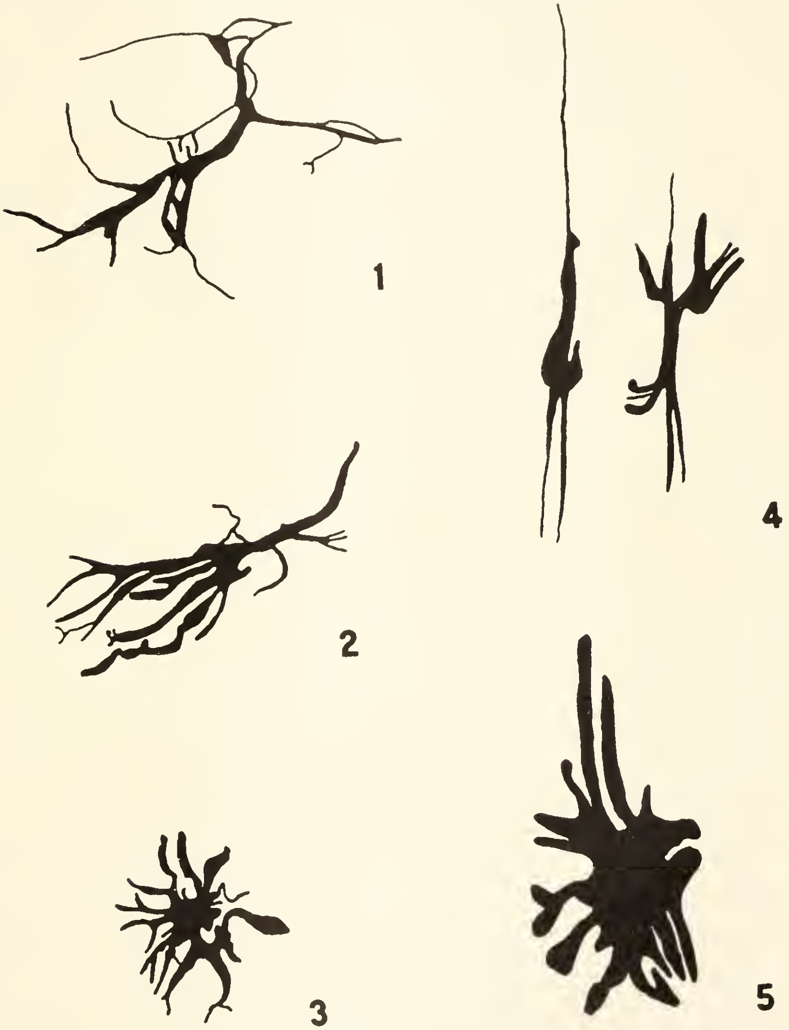


PLATE II

Semidiagrammatic drawings of completely expanded melanophores of the frog in (1) the pleura, (2) the mesenteries, (3) the fascia of the leg muscles, (4) the fascia of the body-wall muscles, and (5) the fascia of the back muscles.

To facilitate a comparison of conditions among the deep-seated melanophores, four conventional stages have been defined as follows: (1) the *punctate* stage, an entirely contracted state in which the cell appears as a minute black dot; (2) the *stellate* stage, or one in which the pigment occupies only the main roots of the cell processes; (3) the stage of *incomplete expansion*, in which the melanin partly fills these; and (4) the stage of *complete expansion*, in which the pigment has spread into the most remote branches (Pl. I).

TABLE I

The variation in the degree of contraction among the melanophores of frogs confined in darkness or on a white illuminated background.

Location of melanophores	Case a74—Kept in dark 20 hours	Case a107—White illuminated surroundings 24 hours	Case a190—White illuminated surroundings 2 weeks
Skin on back	Stellate	Stellate	Stellate
Web	Stellate	Stellate	Stellate
Back muscles	Punctate to stellate	Punctate to incomplete expansion. Mostly stellate.	Punctate
Pleura	Stellate	Punctate	Punctate to incomplete expansion
Pericardium	Punctate to incomplete expansion	Stellate to incomplete expansion	Incomplete expansion
Muscles of body wall	Punctate to stellate	Punctate	Punctate to stellate
Mesenteries	Chiefly punctate to incomplete expansion	Punctate	Punctate

OBSERVATIONS

Frogs

In frogs, the deep-seated melanophores are particularly abundant in the connective tissues of the following regions: the fascia of the leg muscles, of the muscles of the back, of the muscles of the body wall—especially the m. obliquus internus—, the mesenteries, the pericardium, the pleura, and the lining of the sub-dermal lymph sacs. These cells are very similar in general appearance to the melanophores of the skin, with the exception of those in the fascia of the back muscles, which resemble those in tadpole connective tissues (Pl. II).

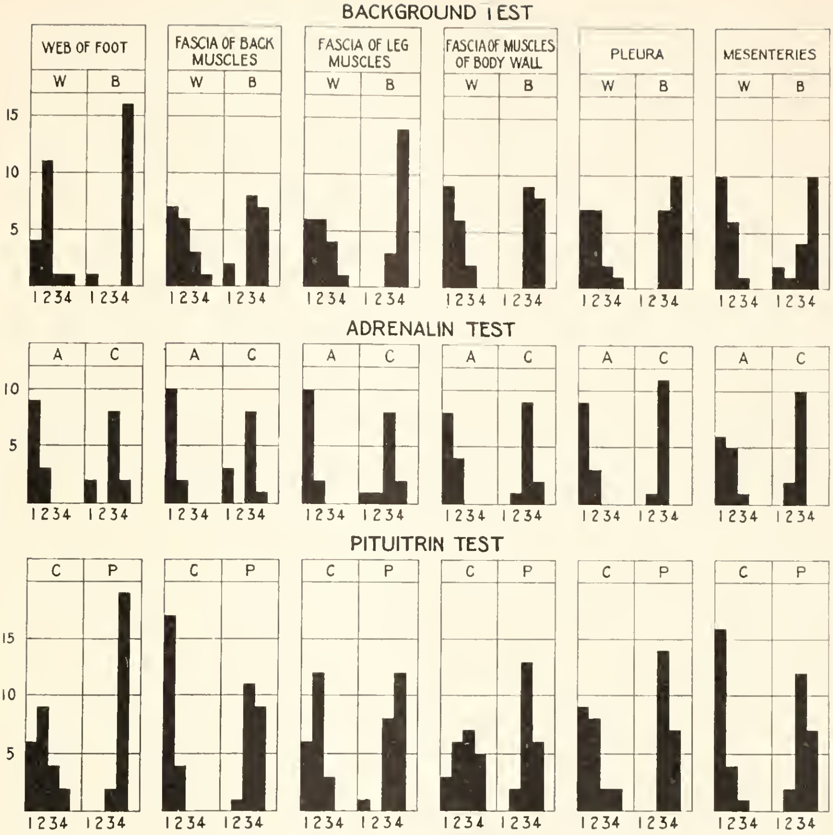


Diagram 1. Tabulation of the effects of background, adrenalin, and pituitrin on the melanophores in six different parts of the frog: the web of the foot, the fascia of the back-muscles, of the leg muscles, and of the muscles of the body-wall, the pleura, and the mesenteries (as shown at the top of the diagram). In the background tests, the shade of the surroundings, white or black, is indicated by *W* or *B* at the head of each column in the upper row. In these tests, as in all the subsequent ones, the results of the factor effecting the more complete contraction are placed to the left and of that producing the less complete to the right in each contrasting pair.

Seventeen frogs were kept on each background for forty-eight hours before observation. In every frog, the amount of melanophore contraction was noted as belonging to one of four stages of contraction of which 1 is maximum contraction and 4 is minimum contraction. The number of cases falling in each class is recorded in black columns over the appropriate figure. Thus, in the web of the foot of frogs on a white background, four animals showed complete contraction, eleven part contraction, one part expansion, and one full expansion: on a black background, one exhibited full contraction and sixteen complete expansion. The remaining tests are all tabulated in a similar manner.

In the adrenalin test, the frogs treated with a 0.5 cc. injection of the drug three hours before examining and their controls with 0.5 cc. tap water injections are designated *A* or *C* respectively at the top of each column in the second row. The twelve frogs in each series were kept on an illuminatd black background for two days before observation.

In the pituitrin tests, the frogs subjected to a 0.5 cc. pituitrin injection three hours before examination and their controls with 0.5 cc. of tap water are distinguished in the diagram by *P* or *C* at the top of each column in the bottom row. The twenty-one frogs in each series were confined in complete darkness for several days before their inspection.

The deep-seated melanophores of frogs confined on a black background for periods extending from a day to a week were predominantly expanded. Yet there were always some cells among them in stages of contraction. This situation was unlike that in the skin where the melanophores were always in a uniform state of expansion.

TABLE II

The rate of response in melanophores to adrenalin. Frogs kept in blackened battery jars under illumination before the injection and until killed.

Location of melanophores	Case a11 1 hr. after 0.5 cc. adrenalin	Case a53 2 hrs. after 0.5 cc. adrenalin	Case a69 3 hrs. after 0.5 cc. adrenalin	Case a112 27 hrs. after 0.5 cc. adrenalin
Skin on back	Punctate	Punctate	Punctate	Stellate
Web	Punctate	Punctate to few stellate	Punctate	Stellate
Leg muscles	Complete expansion	Incomplete expansion. Few punctate.	Punctate	Punctate to incomplete expansion
Pleura	Complete expansion	Incomplete expansion	Stellate	Stellate to incomplete expansion
Pericardium	Complete expansion	Complete expansion	Incomplete to complete expansion	Stellate to complete expansion with proximal concentration
Muscles of body wall	Complete expansion	Incomplete expansion. Some punctate.	Punctate	Punctate
Mesenteries	Complete expansion	Incomplete expansion to few punctate	Punctate	Stellate
Back muscles	Complete expansion	50% punctate, 50% incomplete to complete expansion	Punctate. Occasional stellate.	Chiefly punctate. Some incomplete expansion.

In three individuals placed in a white dish for a day, there was a marked trend toward the contracted state among the internal melanophores. (Table I, third column.) As in the frogs kept on a dark background, there were all stages in the proximal withdrawal of the pigment. Likewise, animals kept on a white background for a week or more showed great variety in the amount of contraction. (Table I, fourth column.) However, a greater general contraction usually existed with the longer period in white surroundings, suggesting a more sluggish response in the internal melanophores. Though not extreme, the contrast in the position of the pigment of these cells was evident in seventeen frogs kept in white dishes for at least two days, and in a like number confined in black jars for a similar period. (Diagram 1, top row.)

Table III

The response of melanophores to pituitrin. See also Table II, fifth column, for condition such as probably existed before the pituitrin injection in a113. Also, see Table I, second and third columns, for the condition of melanophores such as probably existed before the injections of pituitrin in a99 and a105 respectively.

Location of melanophores	Case a113—Pituitrin following adrenalin	Case a99—Pituitrin after 24 hrs. in dark	Case a105—Pituitrin after 2 days on white illuminated background
Skin on back	Complete expansion	Complete expansion	Complete expansion
Web.	Complete expansion	Complete expansion	Complete expansion
Muscles of leg	Incomplete expansion	Incomplete expansion	Incomplete expansion
Muscles of back	Incomplete expansion	Incomplete expansion	Incomplete expansion
Pericardium	Incomplete to complete expansion	Complete expansion	Incomplete expansion
Pleura	Incomplete expansion	Incomplete expansion	Stellate to incomplete expansion
Mesenteries	Punctate to incomplete expansion	Incomplete to complete expansion	Incomplete expansion
Muscles of body wall	Incomplete expansion	Incomplete to complete expansion	Incomplete expansion

Adrenalin, which acts promptly and invariably on the dermal melanophores, also effected a slower contraction of the internal pigment cells. An injection of 0.5 cc. of a 1 : 1,000 solution of adrenalin chloride (Parke, Davis and Company) in the dorsal lymph spaces of eight frogs kept on a dark background for some days to insure expansion of these cells, evoked no reaction in the deep-seated melanophores for more than an hour (Table II, second column). Yet, the same dosage always caused the skin to become extremely light-colored within twenty minutes. While a considerable percentage of the melanophores in the fascia of the back muscles of seven frogs contracted within an hour after an injection of 0.8 to 1.0 cc. of adrenalin, the effect of increasing the dose on the deep-seated melanophores was not pronounced. If, however, two hours elapsed between a 0.5 cc. injection and the inspection of the internal tissues (Table II, third column), as noted in four cases, at times as many as half the melanophores were entirely contracted, with a greater number among the pigment cells in the fascia of the back muscles. On increasing the period between the injection and the subsequent examination to three hours (Table II, fourth column), the contracted phase became general save in the pericardium. Here, the pigment migrated proximally only after many hours, as was noted in three or four frogs kept alive for nearly a day after the injection (Table II, fifth column). The dose of adrenalin was controlled in every case by an equal one of tap water. Frogs so treated never exhibited as great a contraction of the melanophores as those with adrenalin (Diagram 1, middle row). In fact, the stage of expansion was essentially similar to that in frogs kept in black jars without injections.

As an expansion of the melanophores is usual in the laboratory, and as their contraction is brought about so slowly, it was a bit uncertain in the pituitrin tests, whether a contraction of these cells had occurred in the individual frogs before injecting the drug effecting expansion. Since adrenalin was certain to produce a general proximal migration of pigment, it at first seemed desirable to inject it the day before the pituitrin. This procedure produced a complete expansion of the internal melanophores in eleven frogs, three hours after the injection of 0.5 cc. pituitrin (Parke, Davis and Company—" Obstetrical "). As an interaction of the drugs might have caused this, another test became preferable. Darkness was quicker and more certain in obtaining a contraction of the internal melanophores than white illuminated surroundings (Table I, second and third columns). In frogs confined in the dark for several days, a 0.5 cc. injection of pituitrin effected a more or less complete expansion in about three hours (21 cases) (Table III). In controls with the same amount of tap water, the melanophores were predominantly in the punctate or stellate condition (Diagram 1, bottom row).

Tadpoles

In the tadpoles of *Rana clamitans* in their second year, stubby relatively unbranched melanophores are numerous in most connective tissues, particularly in the peritoneum, the mesenteries, the pleura, and the pericardium (Pl. III).

When the tadpoles were confined in black jars for a day or more, the sub-epidermal melanophores almost always (22 out of 25 cases) exhibited a uniform state of maximum expansion. This condition was extended to the deep-seated melanophores in the pleura, the pericardium, the mesenteries, and, to a certain extent, to those of the peritoneum. In the latter tissue, however, in about half the tadpoles, some of these cells were in the stellate condition, while in one case the greater proportion of these was punctate.

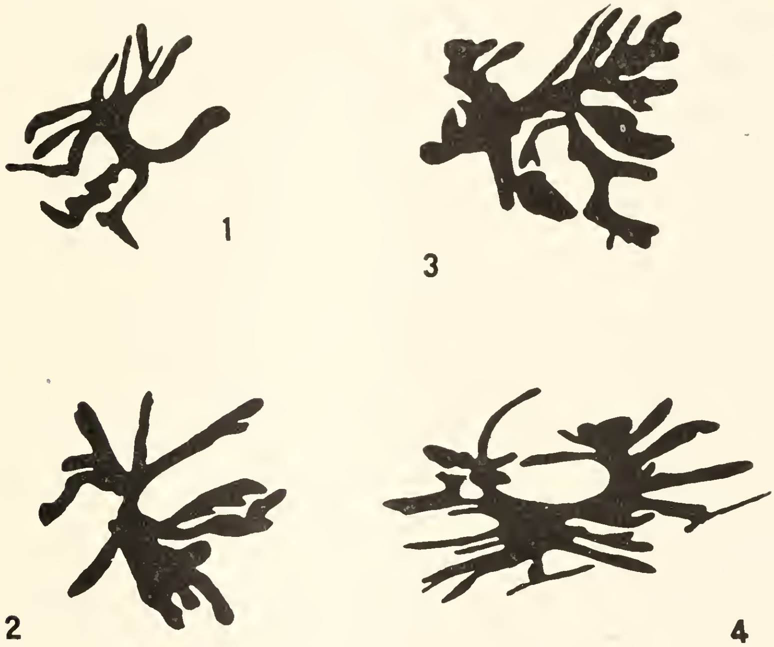


PLATE III

Semidiagrammatic drawings of completely expanded melanophores of tadpoles in (1) the pleura, (2) the mesenteries, (3) the peritoneum, and (4) the pericardium.

If tadpoles were kept in white dishes for a similar period, the subepidermal melanophores were usually (23 out of 25 cases) entirely contracted. Among the internal melanophores the amount of contraction which took place under these circumstances was not so great. Except for the melanophores of the pleura, which were predominantly contracted, there were many which exhibited a more or less distal distribution of pigment (Diagram 2, top row).

In larvæ, whose deep-seated melanophores responded as promptly as those in the skin to changes in background, adrenalin was a slower agent for contracting such cells than in the adult frog. Three hours after a

0.3 cc. injection in a 1 : 1,000 solution into the body cavity, the subepidermal melanophores ranged from the punctate stage to that of incomplete expansion and the internal cells remained for the most part completely expanded. Not until five hours (26 cases) after such a dosage were the majority of the skin melanophores contracted and even then a large percentage were in the stellate condition. Essentially the same situation was found in the internal melanophores. The proportion of animals with these cells in a punctate or stellate condition was greater than in tadpoles on a white background. With longer periods between the injection and the inspection of the animal, no great change occurred in the amount of contraction, and with a larger dose, the animal died within twenty-four hours. In controls with tap water, all the melanophores save a few in the peritoneum of about one-third the animals were completely expanded (Diagram 2, middle row).

With 0.3 cc. of pituitrin injected five hours before examination, the melanophores of tadpoles kept in white dishes were almost always (20 out of 25 cases) in the completely expanded phase. In the controls with tap water, there were only a few (5 out of 25 cases) with completely expanded melanophores (Diagram 2, bottom row).

Fundulus

When compared with the number of deep-seated melanophores in

DIAGRAM 2. Tabulation of the effects of background, adrenalin, and pituitrin on the melanophores in five different parts of the tadpole: the dermis, the pleura, the pericardium, the mesenteries, and the fascia of the body wall muscles (as shown at the top of the diagram).

In the background tests, the shade of the surroundings, white or black, is indicated by *W* or *B* at the head of each column in the upper row. In these tests, as in all subsequent ones, the results of the factor effecting the more complete contraction are placed to the left and that producing the less complete to the right in each contrasting pair. Twenty-five tadpoles were kept on each background for forty-eight hours before observation. In every tadpole, the amount of melanophore contraction was noted as belonging to one of four stages of contraction of which 1 is maximum contraction and 4 minimum contraction. The number of cases falling in each class is recorded in black columns over the appropriate figure. Thus, in the dermis of tadpoles on a white background, 23 showed complete contraction, 1 part contraction, and 1 part expansion: on a black background, 22 exhibited complete expansion, 2 part expansion and 1 part contraction. The remaining tests are all tabulated in a similar fashion. In the adrenalin tests, both the tadpoles treated with a 0.3 cc. injection of the drug five hours before examining and their controls with 0.3 cc. tap water injections are designated *A* or *C* respectively at the top of each column in the second row. The 26 tadpoles in each series were kept on an illuminated black background for two days before observation. In the pituitrin tests, the tadpoles subjected to a 0.3 cc. pituitrin injection five hours before examination and their controls with 0.3 cc. of tap water are distinguished in the diagram by *P* or *C* at the top of each column in the bottom row. The 26 tadpoles in each series were confined in white dishes for several days before their final inspection

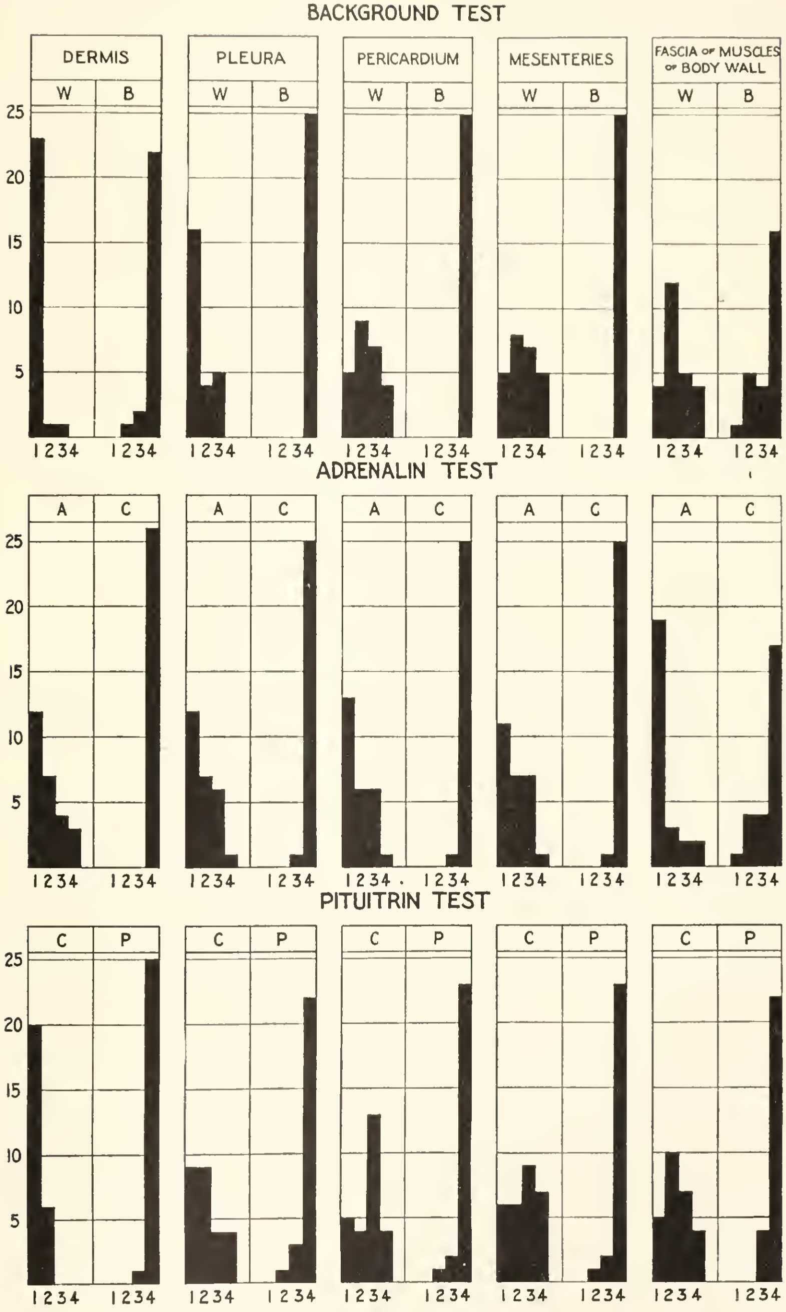

BACKGROUND TEST
DERMIS
PLEURA
PERICARDIUM
MESENTERIES
FASCIA OF MUSCLES OF BODY WALL
W
B
ADRENALIN TEST
A
C
PITUITRIN TEST
C
P
25
20
15
10
5
1234

Diagram 2

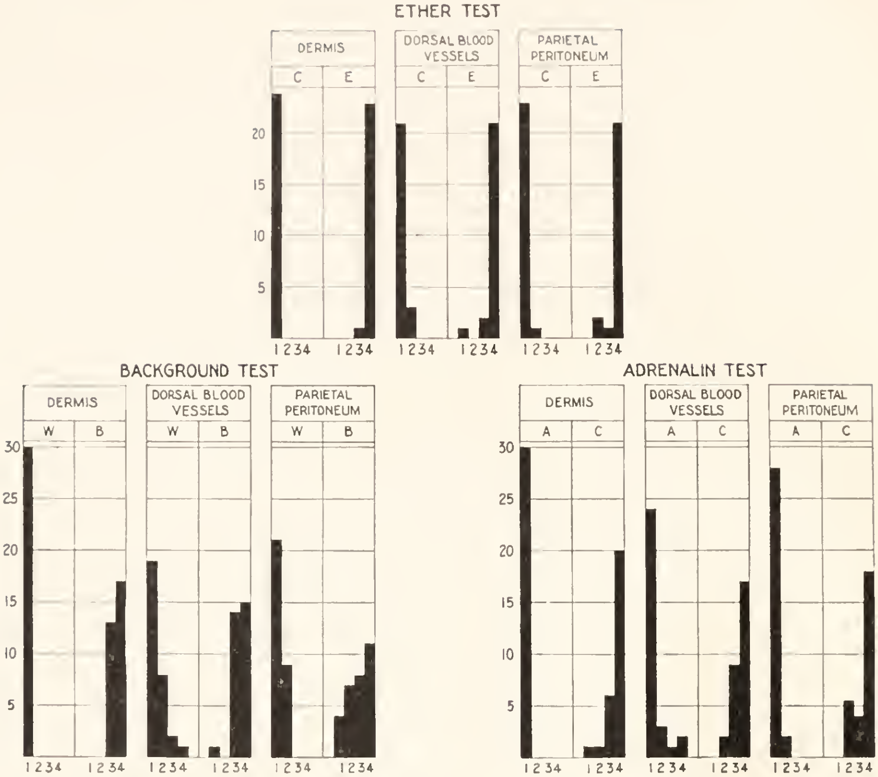


Diagram 3. Tabulation of the effects of ether, background, and adrenalin on the melanophores in three different parts of the killifish: the dermis, the connective tissue covering of the dorsal blood vessels, and the parietal peritoneum (as shown at the top of each column).

In the ether tests, the fish with a few cubic centimeters of this drug added to the water fifteen minutes before their examination and their controls in water alone are distinguished in the diagram by *E* or *C* at the top of each column in the upper row.

In these tests, as in all subsequent ones, the results of the factor effecting the more complete contraction is placed at the left and that producing the less complete to the right in contrasting each pair.

Forty-eight fish were kept in white dishes for a day before observation, of which but half were treated with ether. In every fish, the amount of melanophore contraction was noted as belonging to one of four stages of contraction of which 1 is maximum contraction and 4 minimum contraction. The number of cases falling in each class is recorded in black columns over the appropriate figure. Thus, in the dermis of fish kept on a white background, 24 showed complete contraction: on a white background with ether added to the water, 1 showed part expansion, and 23 complete expansion. All the remaining tests are tabulated in a similar fashion.

In the background tests, the shade of the surroundings, white or black, is indicated by *W* or *B* at the top of each column in the lower left-hand chart. Thirty fish were kept on each background for a day before observation.

In the adrenalin tests, both the fish treated with 0.2 cc. injection of the drug half an hour before examining and their controls with 0.2 cc. tap water injections are designated *A* or *C* respectively at the top of each column in the lower right chart. The 30 fish in each series were kept on an illuminated black background for a day before observation.

amphibia, those in *Fundulus* are scarce. Those in the mid-dorsal part of the parietal peritoneum and in the connective tissue of the dorsal blood vessels are large sheet-like cells. Those in the latero-dorsal portions of the parietal peritoneum and the more dorsal parts of the mesenteries and visceral peritoneum branch radially. In the visceral peritoneum, the true melanophores were usually obscured by melanin-filled syncytial cells—the " Pigmentflecken " of Ballowitz (1920). (Pl. IV.)

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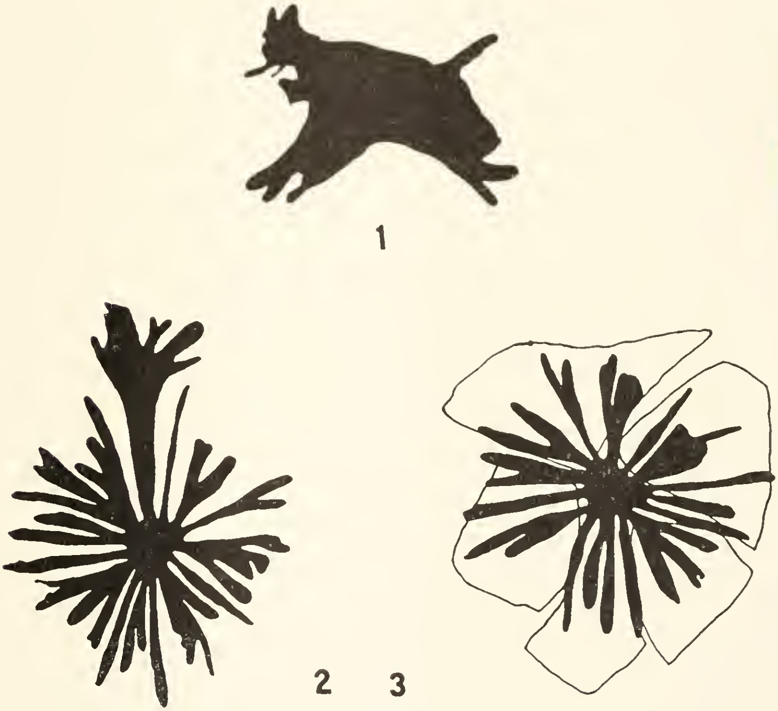

PLATE IV

Semidiagrammatic drawings of completely expanded melanophores of the killifish in (1) the connective tissue covering the dorsal blood vessels, (2) the parietal peritoneum, and (3) the visceral peritoneum (including the syncytial melanin-filled cells in outline).

All the melanophores started to contract when the fish were transferred to white surroundings and a day in them insured complete contraction in the majority of the cells (30 cases). Indeed, expansion of these indicated a slow dissection. Although not always completed for several days, there was an immediate distal migration of the pigment in all the melanophores with a change to the black jars (30 cases) (Diagram 3, lower left-hand part).

Even more rapid changes may be effected by injecting 0.2 cc. of adrenalin into the body cavity or tail muscles. Within half an hour the melanophores were well contracted (30 cases) and this condition persisted even after killing the fish, except in some of the cells in the connective tissue of the dorsal blood vessels (Diagram 3, lower right-hand part).

Pituitrin does not affect killifish melanophores. Hence, ether, which produces an expansion of the dermal melanophores (Wyman, 1924), was used instead. The addition of a few cubic centimeters to the water in which the fish were swimming over a white background, proved to exert the same effect on the internal melanophores (Diagram 3, upper part).

Discussion

The similarity in appearance of the deep-seated and dermal melanophores suggests the occurrence of the same activities in both sets of cells. Nevertheless, earlier work seemed to show that this was seldom the case. Yamamoto (1931), in working on the peritoneal melanophores of a Japanese minnow, obtained little, if any support for a nervous control of such cells. However, since the melanophores which he described and figured were quite different from those in the dermis, they were probably the " Pigmentflecken " studied by Ballowitz (1920) rather than true melanophores, since these are scarce, if not entirely lacking in the peritoneum of many species. These may also be the cells Gilson (1926) observed in adult *Fundulus*. In the newly-hatched larvæ, he noted responses in the true melanophores apparently before they were masked by the development of these syncytial cells, and came to the conclusion that they were under nervous control. Later, in full grown killifish, as the expanded state usually persisted in the internal melanophores despite changes in the external coloring of the fish, he considered that there was little evidence for such control. Thus it appears likely that he actually recorded the inactivity of the " Pigmentflecken." In adult *Fundulus* there are few melanophores in the more dorsal portions of the visceral peritoneum, and these are not easy to find except in fish deficient in pigmentation. For this reason, most of the observations in this report were confined to the melanophores of the parietal peritoneum. In this region, the cells were found to respond synchronously with those of the dermis. These findings, with Gilson's results on the larvæ, make it appear likely that the deep-seated melanophores of the killifish, like those in the skin, are chiefly under nervous control.

In amphibians the earlier work also furnished little evidence for any activity in the deep-seated melanophores, but for quite different reasons. Lieben (1906) and Uyeno (1922) tried the effects of adrenalin by perfusing the tissues with it or by its injection a short time before killing the frog. In some cases they recorded a slight proximal migration of the melanin. It now appears that the cause of this apparent inactivity is due to the unusually slow response of these cells. In my experience, the deep-seated melanophores of frogs did not react for at least three hours and those of tadpoles for nearly five hours. The observations of Lieben and Uyeno were made on animals which had been killed before the application of the drug or soon after its injection. Hence there was time for disintegration of the melanophores to set in before they could have responded to the drug. Indeed, it was natural to suppose that, if the drug calls forth any response in the deep-seated melanophores, it would do so in about the same time as it does in the dermis. Yet this never happens. Does this slower response mean that the dermal and internal cells are not controlled by the same agencies? In view of the fact that all the melanophores of fishes are excited by the same means, it would be peculiar to find that the deep-seated and dermal melanophores of amphibians are activated along different pathways, especially as they all react more or less slowly. The dermal melanophores of animals belonging to this group, unlike those in fishes, are known to be predominantly influenced by humoral agencies (Hogben, 1924). If we assume a similar situation to exist among the deep-seated melanophores, what is the explanation of the delayed response in these cells? Since the activating materials are carried in the circulation, there might be differences in the blood supply, on the one hand, to the dermal and, on the other, to the deep-seated melanophores. Because the skin is an accessory respiratory organ, there is probably plenty of oxygen available there. On the contrary, within the animal, there may be less of it and more carbon dioxide. Some observations have been made on the diverse effects of these two gases (Lowe, 1917; Uyeno, 1922; Wyman, 1924). However, their action is seemingly not a factor, because in the end the responses of the two sets of melanophores are essentially similar. A more likely explanation may be found in the distribution of the blood vessels themselves in the respective parts. While the skin is ramified by a network of veins and arteries, the internal connective tissues are supplied by relatively few vessels. Thus, unlike the skin, the melanophores in the deep-seated portions of the animal are often more remote from the blood supply. Not only would it take longer for the humoral materials carried in the blood stream to reach most of the melanophores on account of the longer portage in the lymph system, but these would also become progressively diluted by the lymph the farther away their source in the blood. The former situation may perhaps be the cause of the slower rate of response of the deep-seated melanophores. The latter, since the cells are not equidistant from the blood vessels, would account for the less extreme and more variable states of expansion found among them. In this way, the differences in the response of the dermal and internal melanophores may be explained. Accordingly, it is probable that the deep-seated melanophores are also controlled by humoral agencies.

The occurrence of similar activities in the dermal and deep-seated melanophores brings up the question of the function of these cells. Many representatives of the lower vertebrates by an expansion or contraction of the dermal melanophores, mimic, to some extent, the shade of their surroundings. This is often interpreted as being a protective measure or an aid in capturing their prey. If this be so, it seems odd, at first, that such changes should also take place within the animal. However, when we consider how sluggish the response is in the deep-seated melanophores of frogs and tadpoles, it is unlikely that the changes in the dermal cells are often reflected in the deeper portions of the animal. Accordingly, it is hard to believe that these cells by reason of their activities are ever of any particular use to the animal. In *Fundulus*, on the other hand, where the internal melanophores may be expected to react synchronously with every fleeting color change in the skin, there are so few true melanophores that it is difficult to conceive of their ever playing a very great rôle. Thus, either on account of their sluggish responses or their scarcity, the activities of the deep-seated melanophores are insignificant as compared with those in the skin. Hence, the fact that the deep-seated melanophores may display many of the activities of those in the skin does not make it necessary to dispense with the usual idea that the function of color change is an adaptive one.

SUMMARY

In frogs, the deep-seated melanophores of the fascia of the leg muscles, of the muscles of the back, of the muscles of the body wall, of the mesenteries, and of the pleura respond to white or black backgrounds, adrenalin, and pituitrin in the same manner as do those in the skin, but about six times more slowly.

In tadpoles, the deep-seated melanophores of the fascia of the body-wall muscles, of the mesenteries, of the pericardium, and of the pleura respond to white or black backgrounds, adrenalin, and pituitrin in the same manner as do those in the skin, but react about ten times more slowly to the drugs.

In killifish, the deep-seated melanophores of the parietal peritoneum and the connective tissues of the dorsal blood vessels respond to white or black backgrounds, adrenalin, and ether synchronously with those in the skin.

It is suggested that the difference in the speeds of response of the deep-seated melanophores in fishes and in amphibians is dependent upon the two methods of control which exist in the dermal melanophores of these two classes—nervous for fishes and humoral for amphibians.

In amphibians, the difference in the speed of dermal and deep-seated melanophores is believed to be due to the distribution of the blood vessels. While the skin is ramified with small blood vessels, the deep connective tissue receives a relatively scanty supply, for while many large veins and arteries pass through this tissue, they give off few branches to these parts. Also, the less extreme and more variable condition found among the deep-seated melanophores is thought to be caused by a dilution of the activating materials by the lymph.

The similarity of response in the deep-seated and dermal melanophores does not reveal what function these cells may serve.

Although the internal melanophores obviously play no part in color changes, neither does their activity interfere with the usual idea that the function of color changes in the skin is an adaptive one, because they are either so few in number that their rôle must be slight (fishes), or so sluggish that they seldom reflect the changes of the skin (amphibians).

BIBLIOGRAPHY

Allen, B. M., 1917. Effects of the Extirpation of the Anterior Lobe of the Hypophysis of Rana pipiens. *Biol. Bull.*, **32**: 117.

Ballowitz, E., 1920. Ueber eigenartige Erscheinungen am Peritoneal-Pigment bei Knochenfischen. *Arch. mikr. Anat.*, **93**: 375.

Ballowitz, E., 1920. Zur Kenntnis des Peritonäalpigments bei Knochenfischen. *Anat. Anzeig.*, **52**: 405.

Fischel, A., 1920. Beiträge zur Biologie der Pigmentzelle. *Anat. Hefte,* Abt. 1, **58**: 1.

Gilson, A. S., Jr., 1926. Melanophores in Developing and Adult Fundulus. *Jour. Exper. Zool.*, **45**: 415.

Hogben, L. T., 1924. The Pigmentary Effector System. Oliver and Boyd, Edinburgh.

Hooker, D., 1912. Reactions of the Melanophores of Rana fusca in the Absence of Nerve Control. *Zeitschr. allgem. Physiol.*, **14**: 93.

Lieben, S., 1906. Ueber die Wirkungen Extrakten Chromaffin Gewebes (Adrenalin) auf die Pigmentzellen. *Centralb. Physiol.*, **20**: 108.

Lowe, J. N., 1917. The Action of Various Pharmacological and Other Chemical Agents on the Chromatophores of the Brook Trout Salvelinus fontinalis Mitchill. *Jour. Exper. Zool.*, **23**: 147.

Ogneff, J. F., 1908. Ueber die Veränderungen in den Chromatophoren bei Axolotlen und Goldfischen bei dauernder Lichtentbehrung und Hungern. *Anat. Anzeig.*, **32**: 591.

Parker, G. H., 1930. Chromatophores. *Biol. Rev.*, **5**: 59, SMITH, P. E., 1916. The Effect of Hypophysectomy in the Early Embryo upon the Growth and Development of the Frog. *Anat. Rec.*, **11**: 57.

UYENO, K., 1922. Observations on the Melanophores of the Frog. *Jour. Physiol.*, **56**: 348.

WYMAN, L. C., 1922. The Effect of Ether upon the Migration of the Scale Pigment and the Retinal Pigment in the Fish, Fundulus heteroclitus. *Proc. Nat. Acad. Sci.*, **8**: 128.

WYMAN, L. C., 1924. Blood and Nerve as Controlling Agents in the Movement of Melanophores. *Jour. Exper. Zool.*, **39**: 73.

YAMAMOTO, K., 1931. On the Physiology of the Peritoneal Melanophores of the Fish. *Mem. Coll. Sci., Kyoto Imperial Univ.*, Ser. B, **7**: 189.

THE CHROMOSOMES OF HABROBRACON

MAGNHILD TORVIK-GREB

(*From the University of Pittsburgh, Pittsburgh, Pennsylvania*)

The parasitic wasp *Habrobracon juglandis* (Ashmead) has, for some years, been a subject of genetic investigations. These investigations have consistently demonstrated that in *Habrobracon*, as in Hymenoptera in general, females come from fertilized eggs and are diploid while males come from unfertilized eggs and are haploid. Occasionally biparental males are produced that inherit from the father as well as from the mother. Genetic evidence indicates that they are diploid and that their daughters are triploid. Rarely, and only in a few stocks, daughters are produced by virgin females.

In 1918 P. W. Whiting made a preliminary cytological study of this wasp. He found that the males were haploid, that the first meiotic division was abortive and that the second was equal. Later Anna R. Whiting (1927) gave a tentative chromosome count of 11 for the male and 22 for the female. She further stated that, " in spermatogenesis of biparental males the first maturation division is abortive, the second apparently equational as in normal haploid males."

Since *Habrobracon* proved to be difficult material for cytological study, and since preliminary work indicated chromosome behavior to be comparable to that of many other Hymenoptera, the work was abandoned temporarily.

Meves, as early as 1904, discussed spermatogenesis in the wasp, *Vespa germanica,* showing the abortive first meiotic division and the equal second division characteristic in spermatogenesis of haploid males of many Hymenoptera.

The cytology of parthenogenetic forms was thoroughly reviewed and a bibliography given by A. Vandel in 1931. In the same year Franz Schrader and Sally Hughes-Schrader published a discussion of " Haploidy in Metazoa " in which they included a summary of haploid forms studied cytologically and an extensive bibliography. More recently (1933) Ann R. Sanderson published a critique of the literature and a bibliography of the cytology of parthenogenesis in Hymenoptera.

Although *Habrobracon* is unfavorable material, it has become increasingly necessary to undertake a cytological study since there are many aberrant types known (diploid males, triploid females and daughters of virgin females as well as male mosaics and gynandromorphs).

The present paper deals with the chromosomes of normal males and females as well as with those of biparental males.

MATERIAL AND METHODS

Gonads of freshly eclosed males and females were used for this study. Early in the work it was thought that male gonads of larvæ or pupæ might show more division figures and a few of these were examined. This material proved to be less favorable than that from adult males. Females were allowed to feed on caterpillars, which they normally parasitize, for a few days before dissection in order to insure their being in an active egg-laying condition.

Wasps were dissected in water, gonads removed and immediately

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EXPLANATION OF PLATES

A Zeiss camera lucida and a Spencer microscope equipped with a Zeiss K30X ocular and a Zeiss oil immersion apochromatic 1.5 mm. objective were used in making the drawings. The original magnification of the drawings was 6,900 diameters, but these were enlarged to 13,800. They have been reduced to one-third in reproduction giving a final magnification of 4,600 diameters.

The chromosome complexes shown in polar views of metaphases in Plates I–III are repeated in Plate IV with chromosomes arranged in decreasing order of magnitude for comparative study. These polar views are from sixteen specimens with drawings corresponding as follows:

Specimen number	Drawings	
	Plates I III	Plate IV
1	5	A 2
2	6	A 1
3	7, 8	A 4, 3
4	15, 16, 17	B 6, 7, 8
5	18	B 3
6	19	B 2
7	20	B 1
8	21	B 4
9	22	B 5
10	27	C 3
11	28, 29	C 1, 2
12	34	D 2
13	35	D 1
14	36	D 3
15	38	E 1
16	39, 40	E 2

Explanation of Plate I

FIGS. 1–14 from normal haploid males.

FIGS. 1–2. Early spermatogonia.

FIG. 3. Later spermatogonia.

FIG. 4. Spermatogonial metaphase, side view.

FIGS. 5–8. Spermatogonial metaphases, polar views.

FIGS. 9–10. Spermatogonial anaphases, side views.

FIGS. 11–13. First spermatocytes. Views of the abortive first meiotic division showing the cytoplasmic bud being pinched off.

FIG. 14. Second spermatocyte metaphase, side view.

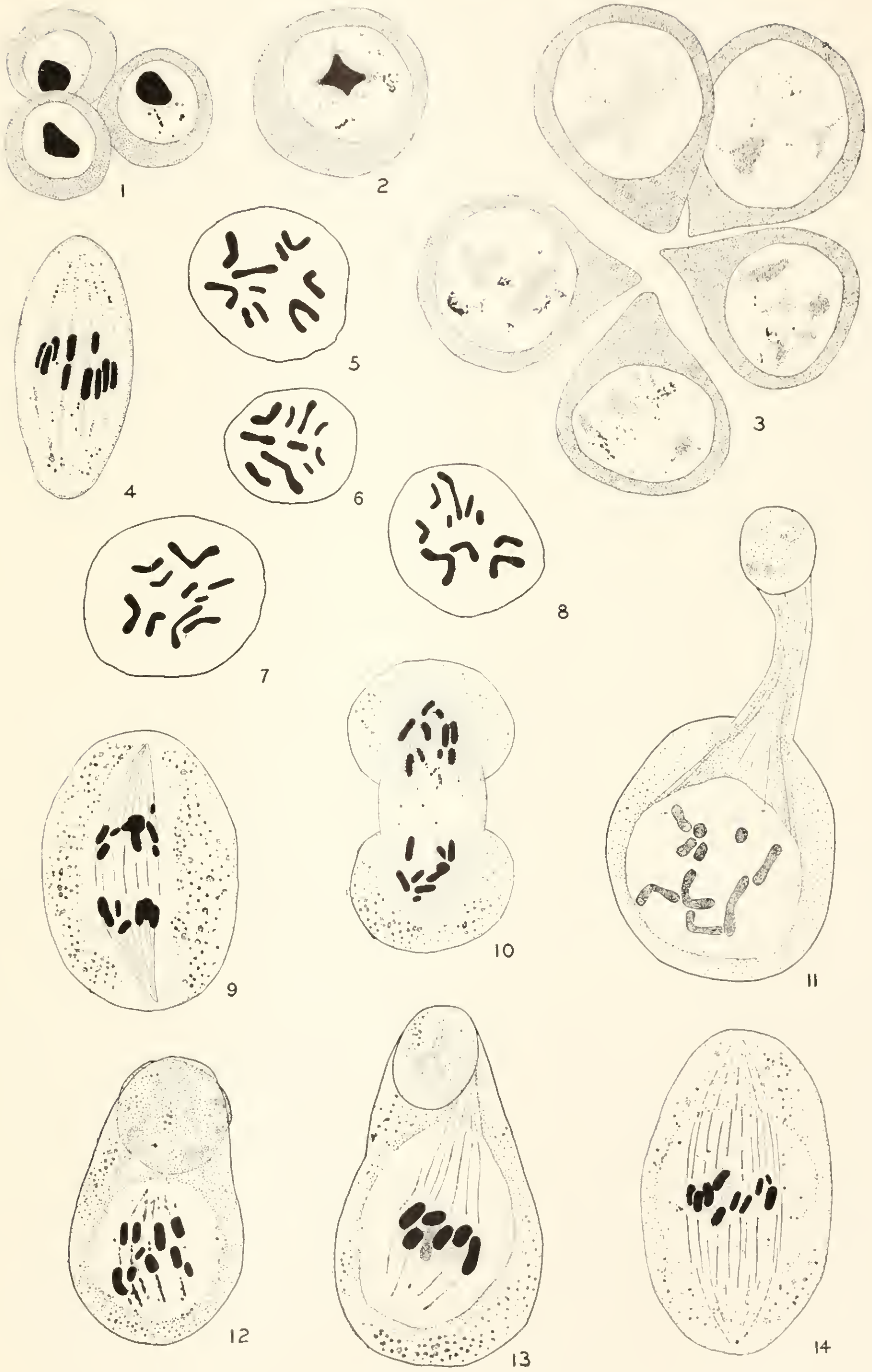
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Plate I

transferred to the fixative. Eleanor Carothers' method for orthoptheran cells was followed in fixing (McClung, C. E., 1929, p. 186). After having been washed and passed through the lower alcohols, gonads were stained *in toto* with eosin in 95 per cent alcohol to guard against possible loss in later handling. They were run through aniline oil and chloroform into paraffin. Sections were cut five to six microns in thickness. Heidenhain's iron hæmatoxylin was used for staining all slides studied—the short method being preferred to the long.

Chromosomes of the Normal Haploid Male

In *Habrobracon* the paired, rounded, yellowish testes (about 1/4 mm. in diameter) are found fused dorsal to the intestine in the posterior part of the body. Each gives off a lateral duct which passes around the intestine and unites with the accessory gland to form a common duct which connects with the penis. Internally the testes are subdivided into a number of cysts. Scattered throughout the testes of immature individuals are spermatogonial cells many of which are in compact rosettes but some are in larger, looser rosettes. In the gonads of late pupæ maturation division figures were observed and even some spermatids, but gonads of freshly eclosed males showed the highest percentage of cells in active division and were, therefore, studied most intensively.

No attempt was made to follow closely the course of development of the spermatogonia. Rosettes of very small cells were observed, growth stages were noted and rosettes of fully-grown cells also were recorded (Plate I, Figs. 1–3).

Good mitotic figures of spermatogonia were rarely seen. In any one testis there was never more than one cyst showing spermatogonial mitoses and in most testes no such divisions were in progress. However, enough figures (from ten different wasps) were found to offer convincing proof of the fact that normally there are ten chromosomes present in spermatogonial cell divisions of the haploid male. The four spermatogonial metaphase plates shown (Plate I, Figs. 5–8) were taken from testes of three different wasps. Counts were made in other cases some of which were below ten, but in such instances plates were slanted or obscured by other cells in the section.

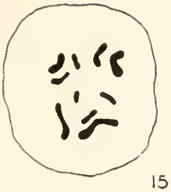
Explanation of Plate II

Figs. 15–22. Second spermatocyte metaphases, polar views, from normal haploid males.

Figs. 23–25. Second spermatocyte anaphase and telophases, side views, from normal haploid males.

Fig. 26. Late spermatogonia from biparental diploid male.

Figs. 27–29. Spermatogonial metaphases, polar views, from biparental diploid males.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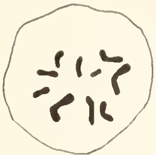
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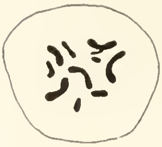
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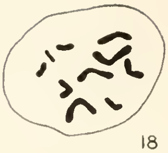
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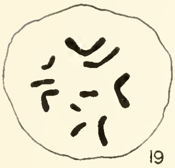
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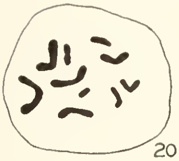
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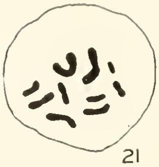
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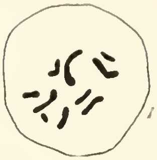
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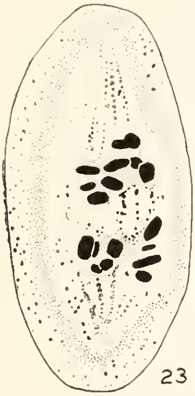
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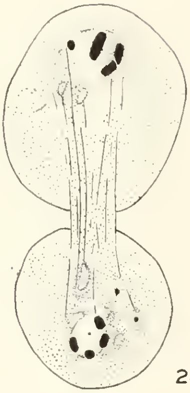
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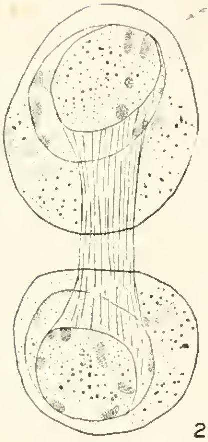
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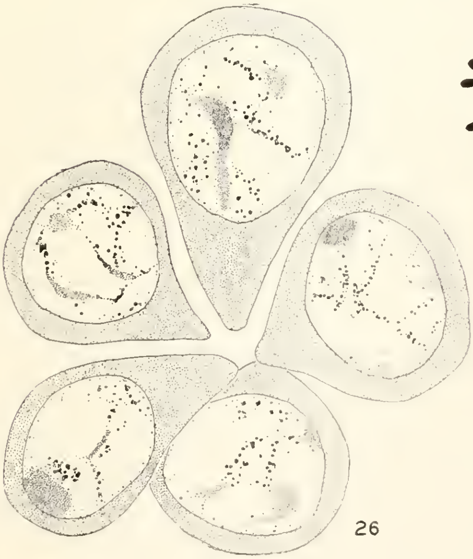
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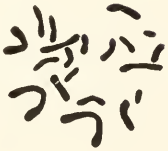
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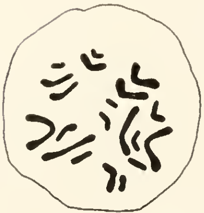
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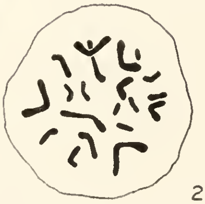
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Plate II

In later prophase stages of the first spermatocyte, after the nucleus has increased in size, the chromatin appears as small bodies lying close to the nuclear wall. Still later the cells are pear-shaped in preparation for the abortive first meiotic division. The chromatin bodies, no doubt condensing chromatin threads, develop into chromosomes which first appear near the nuclear wall and then condense as if in preparation for the division. Meanwhile, a small bud has been cut off at the narrow end of the pear-shaped cell. This bud, which is purely cytoplasmic, soon degenerates (Plate I, Figs. 11–13).

The chromosomes then lengthen into threads but soon condense again and travel to the center of the nuclear mass. The nuclear membrane has now disappeared and the chromosomes remain at the center of the cell where they immediately become aligned on a metaphase plate for the second meiotic division. Among fifty-seven drawings of such plates; forty-seven showed ten chromosomes, seven showed nine, while three had apparently eleven chromosomes. Plates of the two latter groups had questionable regions as did some of the others. However, the best plates consistently showed ten chromosomes. The eight shown in the drawings (Plate II, Figs. 15–22) were taken from sections of testes of six different wasps.

In the second spermatocyte division the cytoplasm divides equally and each chromosome divides. The chromosomes can be counted in some of the anaphase stages but in telophase they are too closely clumped for counting. Following division a nuclear membrane appears, the chromatin opens out and becomes dispersed preceding the formation of the sperm (Plate II, Figs. 23–25).

The stages in transformation of the spermatid were not followed in detail. In an early stage the chromatin appears near the inner surface of the nuclear membrane, first as large granules, later as a heavy band. Elongation of head and tail takes place until finally bundles of spermatozoa are seen, their heads together and their tails usually pointing towards the center of the cyst.

Chromosomes of the Biparental Diploid Male

It was first noted in 1921 (Whiting, P. W., 1921) that occasionally males in *Habrobracon* show paternal traits and, therefore, do not arise

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Explanation of Plate III

Figs. 30–37 from biparental diploid males.

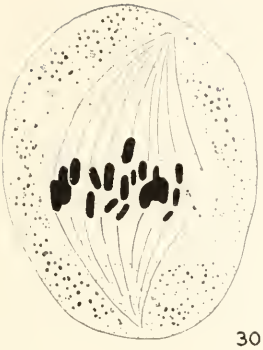
Figs. 30–31. Spermatogonial metaphases, side views.

Figs. 32–33. First spermatocytes. Views of the abortive first meiotic division, showing the cytoplasmic bud being pinched off and the diploid chromosome complex.

Figs. 34–36. Second spermatocyte metaphases, polar views.

Fig. 37. Second spermatocyte telophase.

Figs. 38–40. Oögonial metaphases, polar views, from normal diploid females. Figs. 39 and 40 are from sections of a single cell.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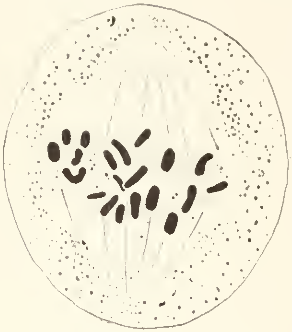
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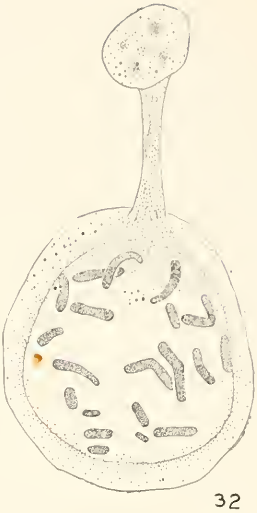
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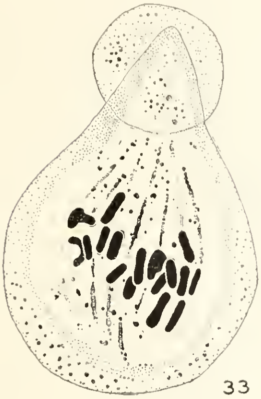
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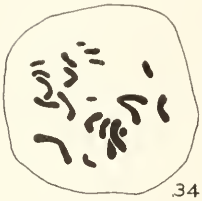
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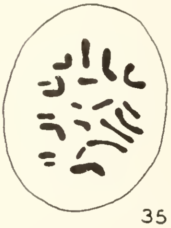
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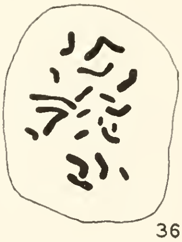
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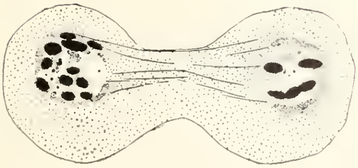
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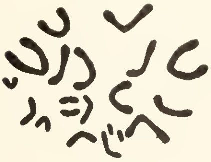
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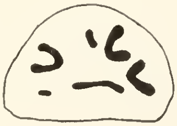
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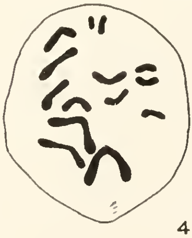
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Plate III

from unfertilized eggs. Subsequent genetic tests have shown that these males occur when related stocks are crossed (Whiting, Anna R., 1925) and that they are diploid (Whiting, Anna R., 1927; Torvik, Magnhild M., 1931).

Cytological evidence of the fact that these males are diploid is now at hand. Cytological observations also show that spermatogenesis follows the same course in the diploid male as in the haploid male. The cells are necessarily larger throughout to accommodate the greater amount of chromatin present but cell growth and cell division proceed similarly in both cases. The figures shown in Plates II and III present some of the more important stages.

Three metaphase plates of spermatogonial divisions (from two wasps) are included (Plate II, Figs. 27–29). These have twenty easily distinguishable chromosomes. There are also three metaphase plates of the second spermatocyte (from three other wasps). Again twenty chromosomes are apparent (Plate III, Figs. 34–36). Among twenty metaphase plates drawn the seven clearest (from six wasps) had twenty chromosomes each; five showed nineteen; four showed eighteen; and four had apparently more than twenty chromosomes. The last no doubt were entering anaphase while the plates giving low counts probably had chromosomes overlying one another in ways which made them indistinguishable. Homologous chromosomes exhibited no great tendency to lie in pairs. By chance they would occasionally lie close to one another. The first meiotic division is abortive and the second apparently equal. Consequently, the sperm formed would be at least approximately diploid, as indicated by genetic tests.

Chromosomes of the Normal Diploid Female

The paired gonads of *Habrobracon* females each consist of two ovarioles morphologically made up of three regions: (1) dorsal, apical end-chamber with oögonia, followed by (2) masses of nurse cells alternating with (3) developing oöcytes each of which is surrounded by follicle cells. Each ovary ends in an enlarged sac or uterus in which mature eggs are contained.

Sections were made of entire ovaries but only oögonial division figures were noted. Metaphase plates were found in ovaries from seven different females. Among ten plates which were drawn six had twenty chromosomes each. The remainder were less clear and, therefore, more difficult to count correctly. Two oögonial metaphase plates are shown (Plate III, Figs. 38–40).

Chromosome Comparisons

The chromosomes of *Habrobracon,* though small, do exhibit differences with respect to size and shape. This was most evident in metaphase plates. In order to make comparisons easier, the chromosomes already shown in views of metaphase plates have been arranged linearly in Plate IV, according to size. In the haploid male there appear to be:

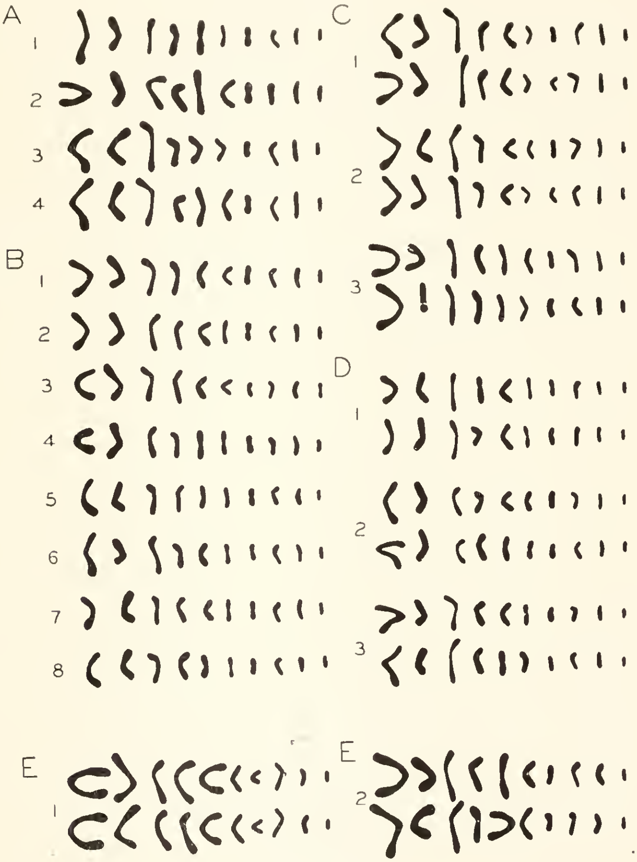

Plate IV

Metaphase chromosome complexes from Plates I–III. From left to right there are shown in decreasing order of magnitude: one *V*, one *L*, one *J*, one *L*, three *V*'s, one *J*, one which is often rod-like but may be a *V*, one rod.

Figs. *A*, 1–4. Spermatogonial complexes of haploid males.
Figs. *B*, 1–8. Second spermatocyte complexes of haploid males.
Figs. *C*, 1–3. Spermatogonial complexes of diploid males.
Figs. *D*, 1–3. Second spermatocyte complexes of diploid males.
Figs. *E*, 1–2. Oögonial complexes of diploid females.

four *V*'s, one of which is always large, two medium to large *L*'s, two *J*'s, one small rod and one chromosome which often is rod-like but may be a small *V*. A study of these figures convinces one of the fact that the diploid group consists of pairs of chromosomes whereas the haploid group contains but one chromosome of each type.

It is interesting to note that the oögonial chromosomes are the largest of those studied. Next in size are the spermatogonial chromosomes of both haploid and diploid males. Spermatocyte chromosomes are the smallest. While they vary somewhat in size, they tend to be thicker and more condensed than other chromosomes observed.

ACKNOWLEDGMENTS

The writer is indebted to Professor P. W. Whiting for suggesting the problem and for guidance. Professor Robert T. Hance generously provided laboratory facilities and help. Dr. G. M. McKinley deserves special mention for aid in obtaining satisfactory lighting facilities. The Committee on Effects of Radiation (National Research Council) has furnished technical assistance and apparatus by grants to Professor Whiting.

LITERATURE CITED

McClung, C. E., 1929. Handbook of Microscopical Technique. Paul B. Hoeber, Inc.

Meves, F., 1904. Ueber "Richtungskörperbilding" im Hoden von Hymenopteren. *Anat. Anzeig.*, **24**: 29.

Sanderson, Ann R., 1933. The Cytology of Parthenogenesis in Tenthredinidae. *St. Andrews Univ. Publ.*, No. 33: 321.

Schrader, Franz, and Sally Hughes-Schrader, 1931. Haploidy in Metazoa. *Quart. Rev. Biol.*, **6**: 411.

Torvik, Magnhild M., 1931. Genetic Evidence for Diploidism of Biparental Males in Habrobracon. *Biol. Bull.*, **61**: 139.

Vandel, A., 1931. La Parthénogenèse. G. Doin & Cie, Paris.

Whiting, Anna R., 1925. The Inheritance of Sterility and of Other Defects Induced by Abnormal Fertilization in the Parasitic Wasp, *Habrobracon juglandis* (Ashmead). *Genetics*, **10**: 33.

Whiting, Anna R., 1927. Genetic Evidence for Diploid Males in Habrobracon. *Biol. Bull.*, **53**: 438.

Whiting, P. W., 1918. Sex-determination and Biology of a Parasitic Wasp, *Habrobracon brevicornis* (Wesmael). *Biol. Bull.*, **34**: 250.

Whiting, P. W., 1921. Studies on the Parasitic Wasp, *Habrobracon brevicornis* (Wesmael). I. Genetics of an Orange-eyed Mutation and the Production of Mosaic Males from Fertilized Eggs. *Biol. Bull.*, **41**: 42.

COMBINATIONS OF CURRENT AND ANTECEDENT CONDITIONS IN RELATION TO WING-PRODUCTION OF APHIDS[1]

A. FRANKLIN SHULL

(*From the Zoölogical Laboratory of the University of Michigan*)

The influence of environmental conditions and genetic or other internal factors upon wing-production in the aphid *Macrosiphum solanifolii* has been repeatedly demonstrated in controlled experiments (Shull, 1928, 1929, 1932). For the most part the environmental factors have been tested singly, while all other agents were kept as nearly constant as possible. This was the best way to demonstrate that a given agent has an influence. In the course of the experiments, however, it became evident that the effect of one factor might easily depend upon the accompanying conditions. It was not merely that the factors often worked in opposite directions, so that their combined effect would be the algebraic sum of their single effects; it was found that the action of one agent might change, not merely in amount but even in sign, in response to changes in other factors.

It became desirable, therefore, to use the several agents in their various combinations. The experiments here described constitute the first exploration of the possible modifying, accentuating, and inhibiting effects. Light, temperature, and the presence or absence of wings in the parent aphids are the chief factors so far tested; and since it would be impossible with the facilities available to use all the known modifying agents, it was decided to use only these three. As a further means of curtailing the labor, only two conditions in each of these fields were employed. The two conditions chosen were such as were known to have different effects, and as would permit a not too slow accumulation of data. With respect to light, the two conditions were continuous light and alternating light and darkness (eight hours of the former, sixteen hours of the latter). As to temperature, 24° and 14° C. were selected; temperatures outside of this range are apt to have deleterious effects. The nature of the parent aphids fell into the two classes, wingless and winged, though it would have been possible and desirable to use intermediate-winged individuals as well.

To these three groups of conditions there was reason to suspect a fourth should be added. This was the set of conditions under which

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[1] This work has been aided by a grant from the National Research Council and the Elizabeth Thompson Science Fund.

the stock of aphids was living from which the parent aphids were drawn for the experiments. While it was desirable that various sets of "stock" conditions be tested (involving light, temperature and wings), no more than one of these could be used within the limits of time and space; and temperature was selected as the condition to be varied in the stocks. Accordingly, more than three years ago one stock was started at 24° C., another at 14° C., both in continuous electric light. Later a third stock was kept at alternating temperatures, 24° during eight daytime hours, 14° for sixteen hours at night. Continuous electric light has been furnished for all these stocks, and daylight was practically excluded. One of the stocks was killed by the breakdown of the constant temperature apparatus, but was at once replaced and soon appeared to give results identical with those of the original stock.

The experiments have started with aphids from each of these three stocks. Some of the aphids from each stock were winged, others wingless. Each kind (winged and wingless) was divided into two groups, one raised at 24°, the other at 14°. At each of these temperatures, some were given continuous light, others alternating light and darkness (eight hours light, sixteen hours darkness). Twenty-four groups of parent aphids were thus necessary for a complete experiment. For some of these groups the conditions under which they were reared represented merely a continuation of their former conditions. For others, they represented a change in temperature, or in light, or in both temperature and light. Only the parents were subjected to the conditions named. Their offspring were brought, in 2-day or 4- or 5-day batches depending on temperature, to room conditions to complete their growth.

Owing to the erratic fluctuations to which wing-production in nearly all aphids seems to be subject, a single experiment of the sort just outlined could not be expected to give reliable results. The experiments have therefore been many times repeated. In this paper are recorded over 167,000 aphids. The rearing of such large numbers has been made possible only by the watchfulness and meticulous exactness of Dr. Helen F. Price; without her aid the experiments must have failed long ago. Over long periods of time the repetitions of the experiments have tended to give constant results; that is, in nearly every test there have been the same kinds of differences between the various groups of offspring. This degree of uniformity in the general results engenders confidence that the contrasts shown by the totals do actually represent the effects of the various combinations tested.

Results of the Experiments

While it would be instructive to give the results of the several repetitions of each test, or even the daily output of each group of parents, in

TABLE I

The number of winged and wingless offspring from parents derived from certain stocks and reared under certain conditions. Data are arranged to show most directly the influence of light.

Parents				Offspring		
		Conditions under which reared				
Winged or wingless	From stock reared at temperature	Temp. ° C.	Light	Wingless	Winged	Percentage winged
Wingless	24°	24	Cont.	3657	3662	50.0
			8–16	3722	1798	32.6
		14	Cont.	4021	3509	46.6
			8–16	4242	881	17.2
	14°	24	Cont.	3309	3315	50.0
			8–16	2474	2482	50.1
		14	Cont.	3207	3952	55.2
			8–16	3750	1081	22.4
	Alt.	24	Cont.	2105	2405	53.3
			8–16	2569	1954	43.2
		14	Cont.	3996	2384	37.4
			8–16	4037	667	14.2
Winged	24°	24	Cont.	4716	7002	59.6
			8–16	3678	2428	39.8
		14	Cont.	9358	7127	43.2
			8–16	12465	3208	20.5
	14°	24	Cont.	5226	2747	34.5
			8–16	5140	1422	21.7
		14	Cont.	5162	1384	21.1
			8–16	2990	439	12.8
	Alt.	24	Cont.	2585	3682	58.8
			8–16	2635	2864	52.1
		14	Cont.	4487	3131	41.1
			8–16	4134	598	12.6
Total				103665	64122	

order to show the fluctuations referred to in the preceding section, space forbids any such detailed presentation. The total numbers are large enough, it is believed, to insure that the fluctuations largely neutralize one another. The data have not been given statistical treatment because it is uncertain what the real unit of expression is. The validity of the contrasts shown seems to be assured by the fact that in most instances the experiments can be grouped, all or most of the experiments in one group showing the same type of contrast. These facts will be fairly apparent on inspection of the data.

TABLE II

The same data as those given in Table I, arranged to show most directly the influence of the temperature at which the parents were reared.

Parents				Offspring		
Reared in light	Winged or wingless	From stock reared at temperature	Reared at temp.	Wingless	Winged	Percentage winged
Cont.	Wingless	24°	24°	3657	3662	50.0
			14°	4021	3509	46.6
		14°	24°	3309	3315	50.0
			14°	3207	3952	55.2
		Alt.	24°	2105	2405	53.3
			14°	3996	2384	37.4
	Winged	24°	24°	4716	7002	59.6
			14°	9358	7127	43.2
		14°	24°	5226	2747	34.5
			14°	5162	1384	21.1
		Alt.	24°	2585	3682	58.8
			14°	4487	3131	41.1
8-16 hr.	Wingless	24°	24°	3722	1798	32.6
			14°	4242	881	17.2
		14°	24°	2474	2482	50.1
			14°	3750	1081	22.4
		Alt.	24°	2569	1954	43.2
			14°	4037	667	14.2
	Winged	24°	24°	3678	2428	39.8
			14°	12465	3208	20.5
		14°	24°	5140	1422	21.7
			14°	2990	439	12.8
		Alt.	24°	2635	2864	52.1
			14°	4134	598	12.6

The total numbers of winged and wingless offspring born of parents derived from the three stocks and reared under different conditions are shown in Table I. As there arranged, it is indicated that wingless and winged parents were taken from each of the three temperature stocks (24°, 14°, alternating); that of each group some were reared at 24°, others at 14°; and that at each temperature some were reared in continuous light, others in alternating light and darkness (eight and sixteen hours, respectively). The offspring from these twenty-four sources are given at the right, with the percentage of winged individuals among them.

Contrast of Light Conditions Under Which Parents Were Reared

The arrangement of the data after any scheme similar to that in Table I is best fitted to contrast the effect of the conditions indicated in the fourth column of the branching tree. In Table I this contrast is between continuous light and alternating light and darkness. Pair by pair the numbers to the right of this column, more particularly the percentages in the last column of the table, show the different results from these two light conditions.

In every pair of experiments except one, regardless of how they differed in other respects, more winged offspring were produced in continuous light than in alternating light and darkness. In the one exceptional pair, the third in the table, the two light treatments had practically identical effects. In one other pair (the eleventh) there would be room to question the significance of the difference if it stood alone. But with every pair excepting one showing a difference of the same sign, and most of them a difference of considerable size, there can be but one conclusion: continuous light in general favors wing-production in this strain of aphids, as against alternating light and darkness.

Contrast of Temperatures at Which Parents Were Reared

The results of the same experiments are arranged in Table II with the temperatures at which the parents were reared placed in the fourth column. This position facilitates comparison of the effects of these two temperatures, since each pair of percentages in the last column of the table shows that contrast directly for one combination of the other factors.

A glance at the right column shows that all of the pairs of percentages show differences of the same sign except one. The first two differences, which include the exceptional one and another which agrees with the majority in sign, are small and are perhaps not significant. The

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rest all indicate that distinctly more winged offspring are produced at high temperature (24°) than at low (14°), no matter what other conditions are combined with it.

TABLE III

The data of Table I rearranged to show most directly the effect of wings or their absence in the parents upon wing-production in their offspring.

Parents				Offspring		
From stock reared at temperature	Reared at temp.	Reared in light	Winged or wingless	Wingless	Winged	Percentage winged
24°	24°	Cont.	Wingless	3657	3662	50.0
			Winged	4716	7002	59.6
		8-16	Wingless	3722	1798	32.6
			Winged	3678	2428	39.8
	14°	Cont.	Wingless	4021	3509	46.6
			Winged	9358	7127	43.2
		8-16	Wingless	4242	881	17.2
			Winged	12465	3208	20.5
14°	24°	Cont.	Wingless	3309	3315	50.0
			Winged	5226	2747	34.5
		8-16	Wingless	2474	2482	50.1
			Winged	5140	1422	21.7
	14°	Cont.	Wingless	3207	3952	55.2
			Winged	5162	1384	21.1
		8-16	Wingless	3750	1081	22.4
			Winged	2990	439	12.8
Alt.	24°	Cont.	Wingless	2105	2405	53.3
			Winged	2585	3682	58.8
		8-16	Wingless	2569	1954	43.2
			Winged	2635	2864	52.1
	14°	Cont.	Wingless	3996	2384	37.4
			Winged	4487	3131	41.1
		8-16	Wingless	4037	667	14.2
			Winged	4134	598	12.6

If, despite their smallness, the differences in the first two pairs are significant, the presence or absence of a *change* in the temperature may be responsible for influencing wing production. In the first pair, those parents which came from a stock reared at 24° and were continued at 24° in the experiments produced the larger number of winged offspring. In the second pair those parents which came from a 14° stock and were continued at 14° produced the more winged offspring. It is possible that mere change from one temperature to another, whether from high to low or from low to high, reduced wing-production somewhat. In any case this could be said only of the wingless parents kept in continuous light. It is difficult to attribute any such effect to mere change in any other part of the table.

Contrast of Winged with Wingless Parents

The data of the experiments are rearranged in Table III in such a way as to show most plainly the effect of wings or their absence in the parents upon wing production in the offspring. This is done by placing the nature of the parents in the fourth column so that the pairs of percentages in the last column will show that particular contrast.

The most striking fact brought out by this arrangement is that the winged parents produced notably fewer winged offspring than did the wingless parents, provided the parents had been taken from the low temperature (14°) stock—but under no other circumstances. It made no difference in what light or temperature they were reared; if only they came from the 14° stock, the winged parents yielded the fewer winged offspring.

When the parents came from the 24° or the alternating temperature stocks, it made less difference whether they were winged or not. Indeed, it might be questioned whether wings made any difference in the offspring. Of the eight contrasts from these two stocks shown in the last column, the winged parents yielded the more winged offspring in six, and fewer winged offspring in two. One of the differences is 9.6 per cent, one 8.9 per cent, a third 7.2, the others less. While it seems likely that this preponderance of the results must indicate that in general wings in the parents favor wings in the offspring when the parents come from high or alternating temperature, the influence can only be slight and is presumably modified by some other factor.

Contrast of Temperatures from Which Parents Were Taken

By placing the temperature conditions of the three stocks in the fourth column the effects of these antecedents are most clearly shown. This is done in Table IV. In that table it is shown, so far as concerns the stocks reared at 24° and 14°, that in every instance more winged offspring were produced by parents taken from the 24° stock than by those taken from the 14° stock provided the parents chosen were winged.

The differences all seem certainly significant. But if wingless females were chosen as parents, then in general more winged offspring were produced by those coming from the 14° stock. There is one exception to this latter statement, namely, the first trio of percentages, in which the wing-production was practically identical for parents taken from both the 24° and 14° stocks.

Table IV

The data of Table I so arranged as to show most directly the effect of the temperature at which the parents and their ancestors were reared (prior to the beginning of experiments) upon wing-production in their offspring, with special reference to the 24° and 14° stocks.

Parents				Offspring		
Winged or wingless	Reared in light	Reared at temperature	From stock reared at temperature	Wingless	Winged	Percentage winged
Wingless	Cont.	24°	24°	3657	3662	50.0
			14°	3309	3315	50.0
			Alt.	2105	2405	53.3
		14°	24°	4021	3509	46.6
			14°	3207	3952	55.2
			Alt.	3996	2384	37.4
	8–16	24°	24°	3722	1798	32.6
			14°	2474	2482	50.1
			Alt.	2569	1954	43.2
		14°	24°	4242	881	17.2
			14°	3750	1081	22.4
			Alt.	4037	667	14.2
Winged	Cont.	24°	24°	4716	7002	59.6
			14°	5226	2747	34.5
			Alt.	2585	3682	58.8
		14°	24°	9358	7127	43.2
			14°	5162	1384	21.1
			Alt.	4487	3131	41.1
	8–16	24°	24°	3678	2428	39.8
			14°	5140	1422	21.7
			Alt.	2635	2864	52.1
		14°	24°	12465	3208	20.5
			14°	2990	439	12.8
			Alt.	4134	598	12.6

It is difficult to see any relation between the experiments performed with parents from the alternating stock and those derived from either of the other two stocks. In some experiments such parents produced more winged offspring than did those from either of the constant temperatures, in other experiments fewer than either, and in still others a number intermediate between those from the two constant temperatures. There appears to be no general rule stating these relations.

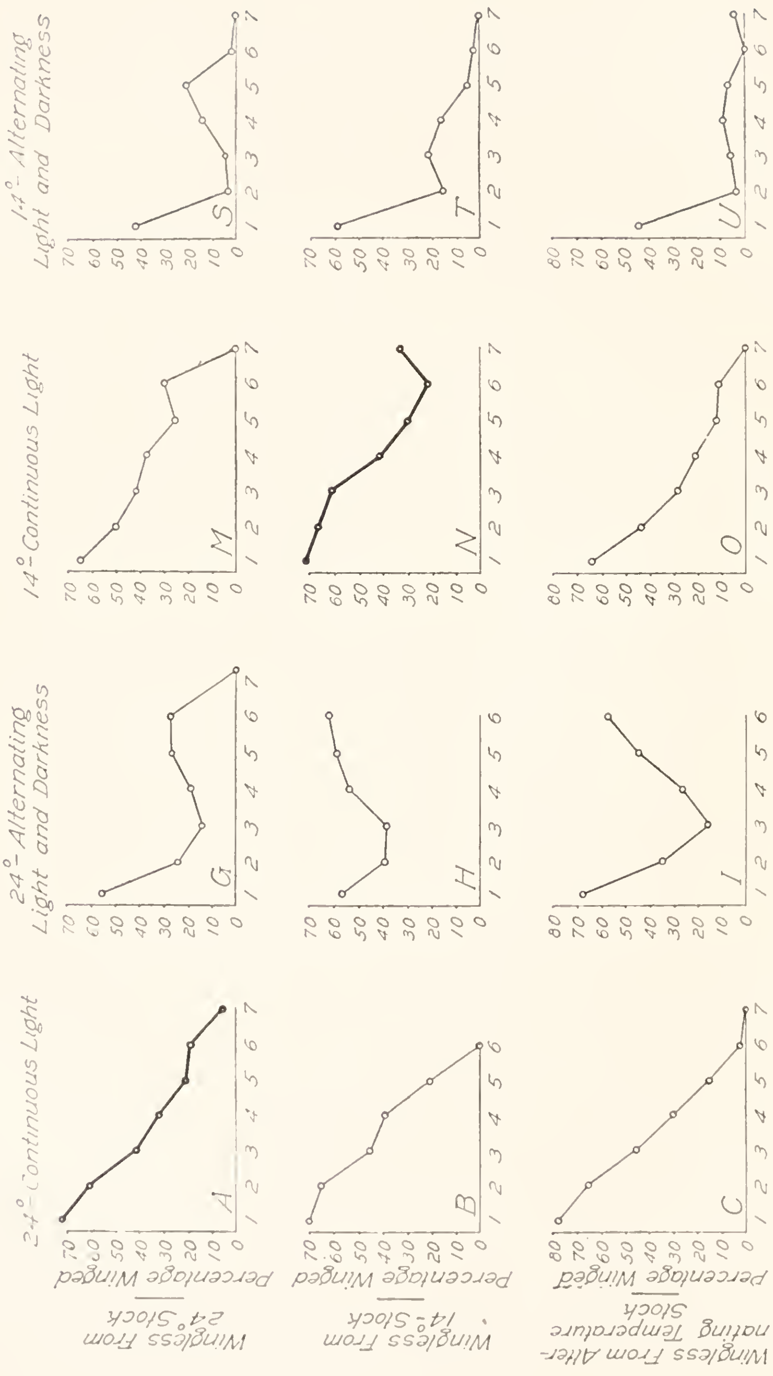
Distribution of Wing Production Through the Family with Respect to Age

It is important in judging the effects of different agents on wing production to know how the wings are distributed among the successive offspring of the treated parents. The experiments were so conducted as to make this information available. In all experiments the parents were removed to the stipulated conditions while in the late fourth instar or just after becoming adult. Their offspring were obtained in successive groups by changing the parents to a new plant every two days if at high temperature, or every four or five days (five in early experiments, four later) if at low temperature. Most families were practically complete in six to eight such successive groups. It is possible, therefore, to ascertain how the proportion of winged offspring changed from the beginning to the end of the family. It would again be instructive to show the families separately, but space forbids. All families derived from a common source and treated in the same way are collected into one lot, just as was done with the data so far presented.

The percentage of winged offspring from the twenty-four lots of parents is shown in the twenty-four curves of Plate I. To save tabular matter the actual numbers of individuals are not given. The total number represented by each curve may be ascertained from Table I. However, the number in each of the six to eight successive lots of offspring does not appear. In general, the early offspring were much more numerous than late ones; the last lot was sometimes so small that a percentage based on it could not be very reliable.

Effect of Age

For some of the groups of parents the conditions of temperature and light at which they were reared represented no change whatever. This is true of curves *A, D, N* and *Q*, which are darkened for ease of selection. Whatever change takes place in the percentage of winged offspring from one of these groups of parents may be looked upon as in some respect an effect of age. It is of interest to find that the change with age is of the same sort in all of them; the winged offspring become irregularly less numerous the older the parents are. The decline of the winged individuals is most rapid among the offspring of wingless parents


24°-Continuous Light
24°-Alternating Light and Darkness
14°-Continuous Light
14°-Alternating Light and Darkness
Wingless From 24° Stock
Percentage Winged
Wingless From 14° Stock
Percentage Winged
Wingless From Alternating Temperature Stock
Percentage Winged
A
G
M
S
B
H
N
T
C
I
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50
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80
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7

PLATE I

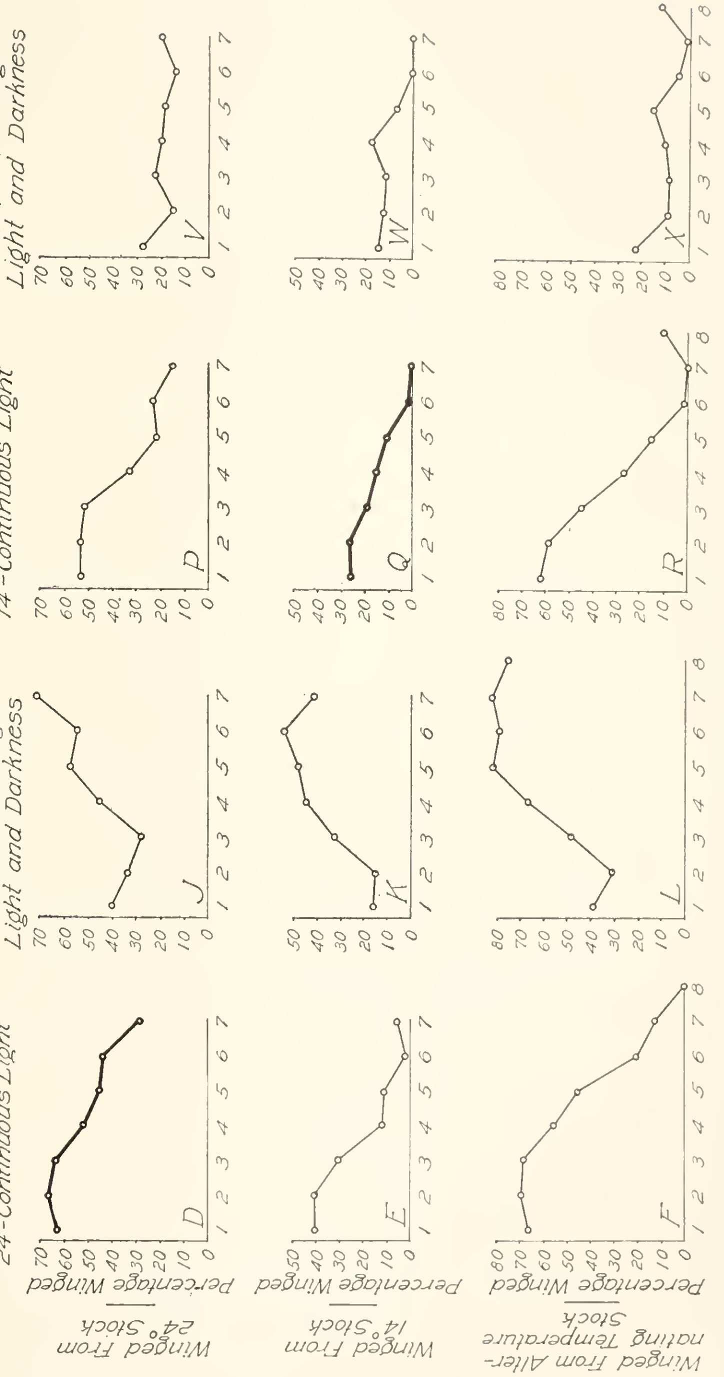

PLATE I—*Continued*

at 24° and continuous light (curve *A*). It is slowest, but unmistakable, among those from winged parents at either 24° or 14° and continuous light (curves *D* and *Q*); however, curve *D* is throughout at a much higher level than *Q*. Whether the decline in curve *N* is greater than that in *A* may be questioned. Except at the beginning, curve *N* is everywhere higher than *A*, but it can hardly be said that the difference increases from left to right; and the sharp rise at the end of *N* is based on only thirty-three offspring in the last 4-day output.

The decline in all these curves, representing an age effect, must of course be taken into account in judging the influence of the other factors. No effect of any other factor is to be inferred unless this result of age is accentuated or reversed or partially nullified or in some way modified in at least part of the family.

Effect of Changed Conditions

All curves other than *A*, *D*, *N* and *Q* represent the distribution of winged offspring through the family as affected by one or more changes of conditions. The first column of curves (*A*—*F*) show the slightest change of any in the entire set of experiments. Curves *B* and *C* do not differ in any striking way from *A*, which means that changing the parents from 14° or alternating temperature to 24° does not modify the distribution of winged offspring as determined by mere age. With respect to winged parents, curve *E* differs from *D* chiefly in being lower; its decline is about the same as that of *E*. The low level of wing-production in *E* is not, however, due to a change of the parents from 14° to 24°, since curve *Q* is even lower. Change from 14° to 24° sufficed to raise curve *E* somewhat, but not nearly so high as *D*. This presumably means that the temperature at which the parents were reared before the experiments began influenced the amount of wing production in their offspring more than did the temperature maintained during the experiments—a statement which applies only to winged parents. Curve *F* differs from *D* only toward the end of the family, where it shows sharply less wing-production than in *D*.

The curves in the second column of Plate I have one striking feature in common, namely, a sharp dip in wing-production about the first one-fourth or one-third of the family. This dip is quite marked when the parents are wingless (curves *G*—*I*), since the initial wing-production, as in most other families from wingless parents, is high. When the parents are winged (curves *J*—*L*) the initial wing-production is quite low, and the dip is less conspicuous or may even disappear. After this depression the curve rises sharply, leveling off or even declining at the end in some. No early depression occurs in any of the curves of the first column (*A—F*); in fact, there is a slight tendency in these curves for the second lot of offspring to cause a hump in the curve, either by including more winged individuals than the first lot does, or by receding less than the later curve as a whole does. These depressions must therefore be attributed to the one factor which is different in curves *G—L* as contrasted with *A—F*, namely, the alternating light and darkness to which the parents were exposed during the experiment.

Curves *M—R*, obtained under a single set of conditions, but from different kinds of parents whose antecedent treatment was various, are not strikingly different. They all show a decline that is mostly attributable to age. There are no constant humps nor depressions. They start high when the parents are wingless, or when the parents are winged and raised previously in alternating temperatures. Their general similarity presumably means that 14° and continuous light during the reproductive period of the parents are more influential than any change to those conditions from any antecedent conditions. The low start of curves *P* and *Q*, particularly the latter, seems to be due to the fact that the parents were winged, though winged parents previously kept in alternating temperatures (curve *R*) did not start out with a small percentage of winged offspring.

Curves *S—X* in the last column of Plate I are very similar. Each presents at the outset a decline which is precipitous if the starting point was high (*S—U*), but moderate if the percentage of winged offspring was at first low (*V—X*). After this decline there is a moderate rise in wing-production, the peak of which comes at various places in the family. Following the rise there is another decline.

Effect of Intermittent Light

The outstanding general result of the various sets of conditions is the effect of alternating light and darkness during the experiments. The second and fourth columns of curves (*G—L* and *S—X*) in Plate I show this effect. A reduction of wing-production in the early part of the family followed by a rise later occurs in every one of these curves. The depth of the early depression depends chiefly on the initial amount of wing-production (which in turn depends chiefly on the presence or absence of wings in the parents), while the height of the subsequent rise depends mostly on the current temperature (high temperature increasing the rise). At high temperature there is little indication of a second fall after the rise, but at low temperature such a decline is present in every instance. It may be plausibly suggested that, if reproduction continued longer at high temperature (*G—L*), there might be a second decline comparable with the one at low temperature (*S—X*).

Possible Interpretations

The results of these experiments show that every factor tested has a very noticeable effect on wing-production. A fair weighting of their effects would undoubtedly assign a greater influence to alternating light and darkness, as against continuous light, during the experiment than to any other agent. Second place would probably be taken by the temperature used (whether 24° or 14°) during the experiment. Third rating would probably go to wings or winglessness in the parents, though its effect is striking only in parents taken from a low temperature stock. And fourth place belongs to the temperature applied to the parents before the beginning of the experiment, the lowest place being assigned to this factor because it acts in a regular and marked way only on the winged parents and only in the two constant temperatures used.

The somewhat rhythmical succession of depressions and peaks of wing-production under the most influential of these agents, namely, alternating light and darkness, indicate that a moderately simple physiological explanation ought to be attainable. Such an explanation should be sought with caution, however. An attempt was made in an earlier study (Shull, 1929) to explain wing-production as due to a substance resulting from the decomposition, in darkness, of another substance produced in the light. The strain then being used for experiments was clone *A* of a later paper (Shull, 1932). As described in the latter paper, clone *A* changed radically in the fall of 1929 to become clone *A'*. It is clone *A'* that furnished the material for the experiments here reported, and wing-production in clone *A'* is in many respects different from that of *A*, even to the extent of directly reversing its response to light. A physiological explanation which fits the results from both *A* and *A'* becomes therefore difficult. It would be possible to postulate curves of physiological change of such shape that their relations to one another could be held to explain most of the facts ascertained in this group of experiments, including the rhythmical change of wing-production in curves *S*—*X* (perhaps also *G*—*L*). Until there is some known physiological feature of the aphids which corresponds to at least part of such assumptions, however, the devising of curves is of doubtful value. It seems the part of wisdom to wait.

SUMMARY

In general, continuous light applied to the strain of aphids here used resulted in more wing-production than did alternating light and darkness, mostly regardless of the other conditions imposed.

More winged offspring were produced at high temperature than at low, in all combinations of other conditions except one. The difference in the one exceptional set of conditions was small.

A mere change of temperature from low to high or from high to low may perhaps reduce wing-production in certain of the combinations of other agents, but not in most of them.

Winged parents produced strikingly fewer winged offspring than did wingless parents if taken from the low temperature stock. Winged parents from the high temperature or alternating temperature stocks produced mostly more winged offspring than did wingless parents, but none of the differences was large.

Winged parents taken from a high temperature stock produced many more winged offspring under all other conditions than did winged parents taken from a low temperature stock.

Wingless parents generally reversed the above response, since they produced more winged offspring if taken from a low temperature stock than if taken from a high temperature, in all combinations of other conditions except one. In that one exception there was no difference between the wingless parents from the two different temperatures.

Regarding the response of parents taken from an alternating stock as compared with constant temperature stocks, no general rule can be stated. The results were very irregular.

Under uniform conditions, and without change from the conditions applied to the parents before their reproductive period begins, there is a rather rapid and steady decline in the number of winged offspring from the beginning to the end of the family. The decline is more rapid for wingless parents than for winged ones.

At high or low temperature and in continuous light the age effect described in the preceding paragraph is the chief factor governing distribution of wing-production through the family.

At high temperature and in alternating light and darkness there is a decline in wing-production early in the family, followed by a sharp rise later, regardless of the type of parents or the temperature from which they were taken.

At low temperature and in alternating light and darkness there is a decline of wing-production early in the family, a slight or moderate rise thereafter, and a decline toward the end of the family, regardless of the type of parents or the temperature from which they were taken.

The most effective of all the agents tested in these experiments is the light conditions (whether continuous or alternating) prevailing during the experiment. Temperature during the experiment is next most important. Wings or winglessness of the parents is third in importance, followed closely by the temperature at which the parents were reared before the experiment began.

The time seems not ripe to attempt a physiological explanation which will fit these results as well as the somewhat divergent ones obtained in previous studies.

LITERATURE CITED

SHULL, A. F., 1928. Duration of Light and the Wings of the Aphid Macrosiphum solanifolii. *Arch. f. Entw. d. Org.*, **113**: 210.

SHULL, A. F., 1929. The Effect of Intensity and Duration of Light and of Duration of Darkness, Partly Modified by Temperature, upon Wing-production in Aphids. *Arch. f. Entw. d. Org.*, **115**: 825.

SHULL, A. F., 1932. Clonal Differences and Clonal Changes in the Aphid Macrosiphum solanifolii. *Am. Nat.*, **66**: 385.

TAXONOMIC AND CYTOLOGICAL STUDIES ON THE CILIATES ASSOCIATED WITH THE AMPHIPOD FAMILY ORCHESTIIDÆ FROM THE WOODS HOLE DISTRICT

I. The Stomatous Holotrichous Ectocommensals

GEORGE W. KIDDER AND FRANCIS M. SUMMERS

(From the Marine Biological Laboratory, Woods Hole, Massachusetts)

This is the first of a series of studies undertaken in an effort to contribute to our knowledge of the ciliates, both commensal and parasitic, that are associated with various invertebrate hosts in the region of Woods Hole, Massachusetts. The present paper deals with the stomatous ectocommensals of three species of the family Orchestiidæ: *Talorchestia longicornis* (Say), *Orchestia agilis* Smith, and *Orchestia palustris* Smith. The study includes not only the morphological details necessary for the determination of the taxonomic rank of this group of ciliates but also a cytological study of the process of binary fission of one of the species.

The material for this investigation was collected in the vicinity of Woods Hole, Massachusetts and the studies carried out at the Marine Biological Laboratory. We wish to express our appreciation to Miss Florence Stuck of Columbia University for calling our attention to the occurrence of some of the ciliates here described.

Material and Methods

Our initial material was collected during the summer of 1931 from Nobska Beach. At this time a number of specimens of the sand flea *Talorchestia longicornis* were taken and transferred in moist sand to New York City where a preliminary study of the ectocommensals was made. During the summer of 1934 intensive collections of the same species of amphipod together with *Orchestia agilis* and *Orchestia palustris* gave us ample opportunity to continue our observations. *Orchestia agilis* and *Orchestia palustris* were also collected from Sippewisset Beach and from the muddy banks of the Eel Pond in Woods Hole.

Talorchestia is found buried in the sands during the day near the high water mark. It is a relatively simple matter to obtain abundant material by digging them out and placing them in a container partially filled with moist sand. *Orchestia agilis* is found in great abundance among the moist and decaying sea weed that has been beached by the tide. They can be collected by the thousands by shaking some of this sea weed over any vessel, care being taken to cover the vessel quickly to prevent their escape. *Orchestia palustris* burrows in the mud under decaying vegetation and must be collected individually. As this species is much less active than the preceding one, abundant material can be collected in a minimum of time.

In all cases the amphipods were brought directly to the laboratory where the preparations of their ectocommensals were made. As the material was abundant, ample opportunity was afforded for a detailed study in the living condition of all of the species of ciliates here described. For the cytological details permanent preparations were made by crushing the host on an albuminized cover glass, the ciliates coming away from the carapace in the body fluids. These smears were then fixed and stained in any desired manner.

For morphological observation the best results were obtained by fixing the ciliates in strong Flemming's fluid, staining in Heidenhain's hæmatoxylin and differentiating in a 10 per cent solution of superoxol, following the method described by Kidder (1934*a*). Excellent results were also obtained by the use of the Borrel mixture following hot (60° C.) Schaudinn's fluid.

For the details of the divisional activity the Feulgen nucleic-acid reaction following hot (60° C.) Schaudinn's fluid proved invaluable, although each stage was checked with material stained in Heidenhain's hæmatoxylin and differentiated in iron alum.

TAXONOMY

Only two genera of holotrichous ciliates are found to be associated as commensals with the three species of amphipod hosts studied. The most abundant ciliates belong to a hitherto undescribed genus, which we have named *Allosphærium* gen. nov., of which there are five distinct species. Less abundant, but occurring regularly, are found three undescribed species of the genus *Chilodonella* Strand.

Allosphærium gen. nov.

Allosphærium is a small (24 μ to 59 μ) holotrichous ciliate. All of the species so far discovered live as commensals on the carapace and gill lamellæ of three species of amphipods (*Talorchestia longicornis, Orchestia agilis,* and *Orchestia palustris*). They are remarkably adapted for this mode of life, being very flat dorso-ventrally and possessing great thigmotactic powers. When washed onto a slide and studied in life it is seen that they adhere tenaciously to the glass or to the surface film of the water. They creep rapidly along these surfaces with a rather steady motion moving in wide circles to the left. When dislodged they swim rapidly in a jerky fashion and quickly settle to any surface with which they come in contact.

When viewed from the dorsal surface these ciliates are nearly oval, although slightly asymmetrical. The left lateral margin conforms more nearly to the anterio-posterior axis than does the right, which is curved in a wide arc. The anterior end is usually slightly pointed while the posterior end is evenly rounded in most cases, although in some it is nearly truncate.

The dorsal surface is arched and devoid of cilia while the ventral surface is slightly concave and covered with from twelve to twenty-seven rows of fine cilia. On the ventral surface the lateral pellicle is extended into two folds partially enclosing the concave ciliated area. The right fold is smooth and extends nearly or wholly the length of the body, while the left fold is more in the form of a lapel, smooth in some cases, notched in others.

The cytostome is located near the anterior end on the ventral surface of the body. It is oval or irregular in shape depending on the species. The posterior border of the cytostome is guarded by a shelf or ridge, perpendicular to the long axis of the body and extending to the left lateral margin. Anterior to this ridge the ventral surface falls away into a trough which forms a naked, rather shallow oral groove. The cytostome is equipped with peculiar specialized ciliary elements. Originating within the oral opening and extending well to the outside are three groups of fused cilia, forming three separate membranes. These membranes are pointed at their distal ends and fan-shaped proximally. Two of them originate from the posterior wall of the cytostome and somewhat overlap one another while the third is always more narrow and originates from the right wall of the cytostome. The ones posterior beat out and up in a direction parallel to the long axis of the body. The narrow lateral membrane beats out and toward the left in a direction perpendicular to the long axis of the ciliate. The cilia of the membranes are fused and beat synchronously as a single unit but the membranes are not synchronized with each other. Their movements are discontinuous and their function is presumably to sweep food into the mouth in much the same manner as the pseudomembranelles of *Kidderia* Raabe 1934*a* (*Conchophthirius*) *mytili* (Kidder, 1933*a*).

There is a single macronucleus, more or less oval in shape, located near the center or slightly anterior to the center of the cell. The single micronucleus is situated just anterior to the macronucleus.

In four of the five species of this genus there are two well-developed contractile vacuoles, one in the anterior fourth of the body well toward the left margin, and one in the posterior fourth of the body, either centrally located or toward the left margin of the cell. The fifth species possesses but one contractile vacuole which is situated toward the left lateral edge just anterior to the cell center.

In the posterior cytoplasm well toward the left side of the body is found a curious inclusion which is constant for all species of the genus. In life this inclusion is in the form of a refractile sphere while in hæmatoxylin preparations it stains intensely. It decolorizes more readily than do the nuclei but can easily be identified, especially in Flemming-Heidenhain preparations. It is negative to the Feulgen nucleic-acid reaction and does not stain with either neutral red or Janus green. The acid (green) component of the Borrel mixture stains it with great intensity. All fixations used, whether or not they contain acetic acid, preserve it. In life as well as in stained preparations this endoplasmic sphere always appears homogeneous as to structure and constant as to shape. Our observations to date have given us no clue as to the possible significance or function of this unique cell inclusion. Because of its behavior during cell division, as will be noted later in this report, we are inclined to regard it as a metaplastid, but one that is very regular in its appearance within the species of this genus.

The five species of the genus *Allosphærium* form a closely integrated group. They differ from one another in average sizes although there is considerable overlapping at the extremes. The number of rows of cilia on the ventral surface is constant for a given species and this criterion, taken with the differences in shape, size, and appearance of the nuclei as well as the ventral pellicular folds, makes it possible to differentiate one species from another readily.

There appears to be little host specificity in this group as all three species of amphipods used in this investigation were found to carry an infestation of at least two of the species of *Allosphærium*.

Following is a short general description of the five species of the genus *Allosphærium*.

Allosphærium palustris gen. nov., sp. nov. (Fig. 1, *A*).—This ciliate is the largest species of the genus so far encountered. It averages 55 μ in length (46 μ to 59 μ). The dorsal surface is weakly arched and possesses three folds. One fold occurs along the right margin in the posterior half. It follows the general contour of the body and terminates near the posterior end of the ciliate. The other two start just posterior to the level of the cytostome near the left margin and end at about the level of the endoplasmic sphere. These dorsal furrows are constant in this species. They disappear only after the ciliate becomes flattened, between the slide and cover, to the extent that only feeble motion is possible.

The ventral surface is nearly flat and covered with twenty-seven rows of fine cilia. Of these twenty-seven rows three originate anterior and to the left of the cytostome, curve around its anterior extremity and follow the contour of the right lateral margin of the body and finally end in an oblique suture near the left posterior edge of the ciliate. The remaining twenty-four rows originate in a line just posterior to the cytostome and proceed backwards. Half of them swing down in an arc to enter the suture from its posterior right side, while the other half either converge at the innermost tip of the suture or enter it from its left anterior side. The cilia in the mid-region of the body are rather short while those of the anterior portion about the mouth and those of the extreme posterior region of the cell are quite long. All of these peripheral cilia beat metachronously.

The cytostome is relatively large, the outlines being regularly notched on the right side. The two posterior ciliary membranes are large and triangular while the right membrane is narrow and long.

The right ventral pellicular fold is very narrow and shallow, extending the full length of the body. The left fold starts just anterior to the center of the cell and is extremely broad. It is smoothly rounded and overlaps a considerable portion of the left posterior region of the ventral surface.

The endoplasmic sphere is always situated just at the inner end of the oblique suture. It is relatively small in comparison with the same inclusion of some of the other species of this genus.

There are two contractile vacuoles, one located just anterior and slightly to the left of the endoplasmic sphere and the other situated near the right border of the cell in the anterior fifth of the body. Their diastolic period is quite long and there appears to be no regularity between the emptying of the two.

The macronucleus is ovoid in outline and appears to be made up of a dense reticulum of chromatin. It lies in the anterior portion of the cell slightly to the right of the center. The single micronucleus is found just anterior to the macronucleus. It is relatively large and spherical and stains very intensely with all of the basic dyes.

Allosphærium palustris is found commonly although never abundantly on the carapace and gill lamellæ of *Orchestia palustris*. In a small number of specimens of *Talorchestia longicornis* a few ciliates of this species were encountered. We have never obtained this species of ciliate from *Orchestia agilis*.

Allosphærium sulcatum gen. nov., sp. nov. (Fig. 1, *B*).—This species is the smallest member of the genus. In length it averages 26 μ, the range being between 24 μ and 32 μ. The most characteristic feature to be noted is the presence of a deep groove or sulcus on the dorsal surface well toward the left margin. This sulcus extends from the level of the cytostome nearly to the posterior end of the organism. It makes a sharp dip to the left near its distal end.

The ventral ciliated surface is somewhat restricted in this species due to the development of the lateral pellicular folds. The right fold encloses approximately one-fifth of the ventral surface while the lapel-like left fold extends over a portion of the endoplasmic sphere. The pellicular folds are continuous around the posterior end in *A. sulcatum*, producing a deep rounded notch at the posterior end of the lapel.

There are only twelve rows of cilia in this species. They are arranged in the same general manner as those of the preceding species. We have never been able to detect the presence of a posterior suture, however, but this may be due to the obscuring effect of the pellicular lapel.

The cytostome is similar in shape and position to that of *A. palustris*. The ciliary membranes are arranged in the same order but the two posterior ones are more attenuated at their proximal ends.

The nuclei and the contractile vacuoles of *A. sulcatum* are similar in relative size and position to those of *A. palustris*.

Allosphærium sulcatum is found regularly but in small numbers on the carapace of *Orchestia agilis* and *Orchestia palustris*.

Allosphærium granulosum gen. nov., sp. nov. (Fig. 1, *C*).—This species is characteristically more rotund than any of the other members of the genus. When viewed from the ventral side its shape is nearly oval and the width of the body is approximately three-fifths that of the length. The smooth dorsal surface is highly vaulted, making this organism quite thick dorso-ventrally. The ciliated portion of the ventral surface is nearly flat. The position and extent of the ventral pellicular folds are like those of *A. sulcatum*, although the left lapel does not extend quite to the endoplasmic sphere.

The ventral surface possesses seventeen rows of cilia originating as

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FIG. 1. All figures are composite drawings from living and stained material, drawn from the ventral side. × 1500.

A. *Allosphærium palustris* gen. nov., sp. nov.
B. *Allosphærium sulcatum* gen. nov., sp. nov.
C. *Allosphærium granulosum* gen. nov., sp. nov.
D. *Allosphærium caudatum* gen. nov., sp. nov.
E. *Allosphærium convexa* gen. nov., sp. nov.
F. *Chilodonella hyalina* sp. nov.
G. *Chilodonella rotunda* sp. nov.
H. *Chilodonella longipharynx* sp. nov.

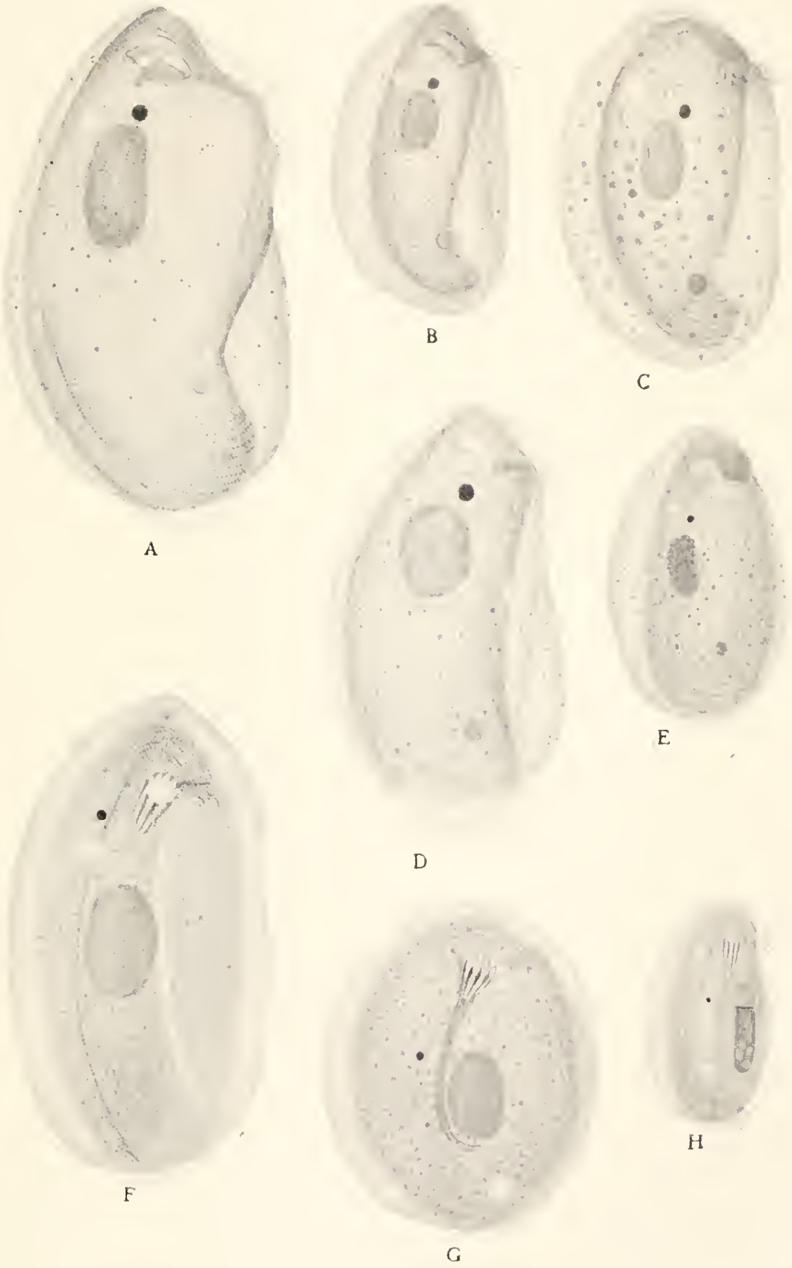

Figure 1

in the preceding species. Here again we were unable to detect the presence of a posterior suture probably because of the overfolding lapel.

The cytoplasm of *A. granulosum* is characteristically filled with large granules that render this organism semi-opaque. These granules together with the thickness of the body enable the observer to identify this species with ease.

The nuclei, contractile vacuoles, and cytostomal structure are practically identical with those of *A. sulcatum.*

Allosphærium granulosum averages 38 μ in length, the extremes being between 32 μ and 42 μ. It is found regularly in small numbers on the carapace of *Orchestia agilis* and *Orchestia palustris.*

Allosphærium caudatum gen. nov., sp. nov. (Fig. 1, *D*).—This species resembles *A. palustris* in general body outline. The ventral surface is slightly concave and possesses fourteen rows of cilia. The dorsal surface is weakly arched. A sulcus runs obliquely from near the center of the right margin to the left posterior edge of the organism. Another shallow furrow starts at the level of the cytostome on the left cell border and continues posteriorly nearly the length of the body. It bends somewhat to the left near its posterior extremity. The bend is exactly the reverse of that noted in the case of *A. sulcatum.*

The pellicular fold of the right ventral margin is narrow, as in *A. palustris,* and is of similar extent. The left fold is quite wide, starting at the level of the cytostome and extending to the posterior end. There is no notched appearance in the left fold, the border of which curves slightly to the right at its posterior extremity.

The nuclei resemble those of *A. palustris* except that the micronucleus is somewhat larger and the macronucleus is more spherical than ovoid. There is but one contractile vacuole located well toward the left border of the cell and slightly anterior to the center. This is the only case so far encountered in this genus where there is a single contractile vacuole.

The cytostome and its ciliary apparatus are almost identical with those of *A. sulcatum* and *A. granulosum.*

The most characteristic feature of this species and one that enables the observer to recognize it with ease is the condition of the posterior ectoplasm. Across the posterior end the ectoplasm is drawn out into a shelf. This caudal shelf is very transparent and does not stain with any of the dyes. The posterior cilia, however, are very long and extend under the shelf so that in stained preparations (Heidenhain's hæmatoxylin) the shelf resembles a little fan.

Allosphærium caudatum is found sparsely on the carapace and gill lamellæ of *Orchestia agilis.* We have never encountered it on either of the other two amphipods used for this study. It averages 41 μ in length, the extremes being 35 μ to 45 μ.

Allosphærium convexa gen. nov., sp. nov. (Fig. 1, *E*).—Because of its regular occurrence and abundance on *Talorchestia longicornis* we have designated this as the type species of the genus in spite of its small size.

As viewed from the ventral side this species is nearly egg-shaped, the narrower end being the anterior. The ventral surface is weakly concave and is supplied with seventeen rows of cilia, arranged in a manner similar to those of the preceding species. Both the right and particularly the left ventral pellicular folds are so narrow that practically the whole ventral surface is exposed. Here the posterior suture is clearly visible. It is an oblique line running anteriorly and to the right from the small notch at the junction of the right and left folds to the endoplasmic sphere.

The cytostome of *A. convexa* is proportionately smaller than any of the other species and is in the form of a nearly circular opening. It is supplied with the usual three ciliary membranes closely resembling those of *A. palustris* as to shape and distribution.

The macronucleus is bean-shaped and lies somewhat to the right near the middle of the body. It is smaller than the macronuclei of any of the preceding species. The micronucleus is minute and occupies a position just anterior to the macronucleus.

There are two contractile vacuoles, the anterior one in the usual position toward the right margin of the cell but the posterior one is found at about the mid-line of the body to the right of the endoplasmic sphere.

Allosphærium convexa averages 29 μ in length. The extremes encountered were from 24 μ to 36 μ. It is found in great abundance on the carapace and gill lamellæ of *Talorchestia longicornis*. We have never encountered it on either *Orchestia agilis* or *Orchestia palustris*.

Chilodonella Strand (Chilodon Ehr.)

Associated with the various species of the genus *Allosphærium* on the carapace of the three amphipods used in this investigation are to be found three species of the genus *Chilodonella*. A large percentage of the hosts examined were found to harbor a few of these ciliates. They are all sufficiently different from previously described species of the genus, as far as we have been able to determine from the literature, to warrant short descriptions. For some time we were in doubt as to the commensal nature of these ciliates, thinking they might represent vagrant free-living forms. A search of the sand and sea weed failed to disclose any ciliates of like nature, however, and this fact together with the general similarities of the *Chilodonella* and the *Allosphærium* convinced us that we were dealing with forms that normally lived as commensals on the carapace of the amphipod hosts. They live but a short time in sea water when washed free from their host, a characteristic of all species of the genus *Allosphærium.*

Chilodonella hyalina sp. nov. (Fig. 1, *F*).—This species is flattened ventrally and convex dorsally. The dorsal surface bears a longitudinal ridge that extends from near the anterior end and curves toward the right to the posterior end. The lateral margins of the ciliate are extended into a hyaline shelf which reminds one of the brim of a hat. This shelf extends completely around the body except for a short space at the extreme posterior end. It is quite thin and seems to be entirely without visible structure.

The cilia are confined to the ventral surface, as in all members of this genus. They are arranged in twenty-three rows. Nine of these rows originate from the anterior side of a line that extends from the mouth to the left margin of the ciliated part of the ventral surface. These nine rows pass anteriorly and to the right, form an arc above the mouth and then pass down the right margin of the cell to end well over toward the left posterior edge. On the left side of the ciliated area eight rows of cilia originate from the posterior side of the short transverse line. They pass backward and bend slightly to the left. The remaining six rows appear to originate from the region of the mouth. There is a sharp line of demarcation between the eight rows of the left side and the rest of the rows of the ventral surface. These two areas are separated by a naked band that extends from the mouth to the posterior end. In life this band is clearly visible as a light streak separating the ciliated area into two parts. Along the line that extends from the mouth transversely to the left margin arise a row of large, stiff cilia. These cilia beat much more leisurely than do the rest of the body cilia.

The mouth is surrounded by a ring of trichites forming a complete pharyngeal basket. The basket is quite short in this species and extends obliquely into the endoplasm. The trichites taper toward their inner ends and disappear from view among the endoplasmic granules. As in the free-living *Chilodonella,* the pharyngeal basket stains deeply with hæmatoxylin. It is also very conspicuous after the Borrel stain where it takes the acid component (green).

The macronucleus is centrally located. It is oval and is relatively large. The chromatin is evenly dispersed in fine granules without any trace of an "endosome" (see MacDougall, 1925, for a description of this body in *Chilodon uncinatus*). The micronucleus is located just to the right of the pharyngeal basket.

There are two contractile vacuoles, one just below the micronucleus and the other near the posterior end to the left of the naked band.

Chilodonella hyalina averages 40 μ in length. The extremes are between 36 μ and 47 μ. It is found exclusively on the carapace of *Orchestia agilis*.

In life *Chilodonella hyalina* resembles to a marked degree the various species of the genus *Allosphærium* and without the aid of the oil immersion lens to note the pharyngeal basket it might well be mistaken for a member of that group.

Chilodonella rotunda sp. nov. (Fig. 1, *G*).—The ventral surface of this species is flat but it is strongly arched dorsally. In side view the organism resembles a derby hat with a very narrow brim, while from the ventral surface it appears nearly round. The brim is not hyaline, as it is in the preceding species, but is somewhat granular.

The cilia are arranged much in the same manner as those of *C. hyalina* but the rows are not separated into two groups. There are twenty rows, five of which originate from the anterior side of the transverse oral line.

The pharyngeal basket is quite different from that of *C. hyalina*. It is composed of trichites that show distinct thickenings near their anterior ends. The basket itself is flared out around the mouth. It narrows rapidly into a long gullet supported by the distal ends of the trichites. The gullet extends into the posterior fourth of the body, curving sharply to the left.

The macronucleus is ovoid and is densely and homogeneously granular. It always lies a little back of the center within the curve of the gullet. The micronucleus lies near the center of the cell to the right of the gullet.

There is a single large contractile vacuole very near the midline at the posterior end of the body. The diastolic period is quite long.

Chilodonella rotunda averages 29 μ in length. The extremes were found to be from 27 μ to 34 μ. It is nearly as wide as it is long. So far we have found it only on the carapace of *Orchestia agilis* and never more than two or three specimens on each host infected. It is easily recognized by its general shape, the shape of the pharyngeal basket, and the dark coloration due to the thickness of the granular endoplasm.

Chilodonella longipharynx sp. nov. (Fig. 1, *H*).—This is the smallest representative of the holotrichous ectocommensals found on this group of amphipods. Its reduced size together with its almost crystalline clearness makes it an exceedingly difficult organism to study in life. In material stained in Heidenhain's hæmatoxylin or the Borrel stain, however, the details of its structure can easily be observed.

The ventral surface is naked except for four rows of rather long cilia. These rows originate anterior to the transverse oral line and describe a semi-circle, up and along the left margin, across the anterior end, down the right margin and thence left across the posterior end. The cilia that originate near the transverse oral line are quite long and relatively thick. As in *C. hyalina* they beat more slowly than do the rest of the cilia along the four rows.

The mouth is surrounded by an extremely long pharyngeal basket which extends nearly to the posterior end of the body. It is made up of straight trichites, which render the basket cone-shaped. In hæmatoxylin preparations the pharyngeal basket is the most conspicuous structure in the cell.

There are two contractile vacuoles both lying to the right of the pharyngeal basket. One is just below the level of the cytostome while the other is in the posterior fifth of the body.

The macronucleus is an elongate oval and is situated along the left margin of the cell. In the majority of ciliates of this species examined there appears a band, very similar to the Kernspalt of numerous hypotrichous forms, extending across the macronucleus. The chromatin on either side of the band is of distinctly different structure, the anterior chromatin being finely granular and faintly staining while the posterior chromatin is made up of a coarse reticulum and stains deeply. The band itself is made up of two parts, a clear plane and an intensely staining plane. This structure is obviously bound up with some stage of development of the ciliate, probably with binary fission. We have found stages where the band was very near to the anterior end of the nucleus and in all gradations of position to about the posterior sixth. Whether the band passes off the end of the nucleus, as it does in a number of hypotrichous forms (see Summers, 1935), or not we cannot say. Although we have hundreds of organisms of this species stained, we have not observed the actual fate of the band. As the band reaches about the halfway point in the nucleus, a deeply-staining sphere is differentiated in the finely granular (reorganized?) portion. This sphere increases in size until its diameter reaches about one-fourth the width of the macronucleus. We cannot say what is the significance or fate of this sphere. We are aware of no other species of *Chilodonella* that shows this band, although it is identical with the one found in *Trochilia (Dysteriopsis) minuta* described by Roux (1901) and Penard (1922) and seems to be similar to those of a number of species of *Chlamydodon* described by Kahl (1931).

The single micronucleus is very small and is always situated in about the center of the cell on the opposite side of the pharyngeal basket from the macronucleus.

Chilodonella longipharynx averages 19 μ in length, the extremes being from 17 μ to 21 μ. It is found most abundantly on the carapace of *Talorchestia longicornis* and less frequently on *Orchestia palustris.* We have never encountered it on *Orchestia agilis.*

Discussion of Taxonomic Affinities

The genus *Allosphærium* obviously belongs to the sub-class Holotricha Stein (Calkins, 1933) and by virtue of the membranes in the mouth must be included in the order Hymenostomida Hickson (Calkins, 1933). But the allocation to any of the established families of that order offers grave difficulties. In none of the members of the order so far described does there occur a restriction of the cilia to the ventral surface. It seems inescapable, therefore, that a new family should be erected for the reception of the genus *Allosphærium.* We are loath to do this at this time, however, because we feel that the affinities for at least one other form that has previously been described are too close to be disregarded, and as yet the existing description is too fragmentary to warrant an analytical comparison. The form in question is *Lophophorina* Penard 1922. This genus, erected for the reception of a single species (*L. capronata*), resembles *Allosphærium* in size, shape, distribubution of cilia, movements and location on its host (fresh-water *Gammarus*). Indeed we were at first of the opinion that we were dealing with species of the same genus as described by Penard. But when it was seen that our forms all conformed so closely to a set pattern which was different in many important respects from *Lophophorina* we were forced to conclude that we were dealing with a new genus. Penard (1922, p. 96) was unable to locate the mouth but he assumed it to be in the anterior portion of the cell near the long " tentacle." By this it can be readily seen that no diagnosis as to its order is possible without knowledge of so important a structure as the cytostome. Still we feel that there is a possibility that when *Lophophorina* is re-investigated it may be possible to erect a family for the reception of both genera (*Lophophorina* and *Allosphærium*), in which case the family name should be, because of priority, Lophophorinidæ. For the present, therefore, we wish to defer the action of establishing a new family.

The previously described species of the genus *Chilodonella* that resemble most closely those found by us on the amphipods studied are those of Penard (1922). His *Chilodonella* (*Chilodon*) *capucinus* (p. 92) possesses two contractile vacuoles as do our *C. hyalina* and *C.*

longipharynx. It has a spherical macronucleus and the cilia are divided into two areas. There are only ten ciliary rows, however, (five right and five left) as contrasted with the twenty-three rows of *C. hyalina.* The shape of the macronucleus and the ciliation set it apart from *C. longipharynx* while the possession of ten ciliary rows as opposed to twenty for *C. rotunda* together with the difference in contractile vacuole number show it to be distinct from the latter species. *Chilodonella* (*Chilodon*) *granulatus* (Penard, 1922, p. 95) has the same type of ciliation as *C. longipharynx* but there are from five to seven rows. The pharyngeal basket is short and re-curved and the cell possesses but one contractile vacuole. Both *C. capucinus* and *C. granulatus* are ectocommensal on *Asellus* and *Gammarus.*

It should be pointed out that the general shape of the holotrichous ectocommensals of both the amphipods and isopods that have so far been described is singularly well adapted for their environment. They are all small flat forms and possess ventrally placed thigmotactic cilia (*Chilodonella, Trochilia, Allosphærium*). When one considers the forces, mainly in the form of water currents, to which they must be subjected and which would tend to effect their removal from the carapace of their various hosts, it is seen that the flatness of their bodies and the adhesive powers of their ventral cilia are of absolute necessity. Existing under the same conditions, it is perhaps not surprising that representatives of two orders of ciliates exhibit convergence to such a degree as to render them practically indistinguishable one from the other except under extreme magnifications.

Division of Allosphærium convexa gen. nov., sp. nov.

Although we have encountered cases of binary fission in practically all of the species described above, we have been able to trace the process completely in only one form, *Allosphærium convexa.* This species occurs in relatively large numbers and we have had an opportunity to study many hundreds of specimens. Of these a rather high percentage were in some phase of binary fission.

Our observations on the process of division of this minute ciliate are confined mainly to the behavior of the macronuclear chromatin. The cytoplasmic structures and the micronucleus are too small to permit us to follow all of the details of their divisional activity.

The chromatin of the macronucleus appears as a dense reticulum during the resting period (Fig. 2, *A*). It stains intensely with all of the nuclear dyes. With the Feulgen nuclei-acid reaction the reticulum appears to enclose many vacuole-like spaces of varying sizes.

The first sign of fission is to be seen in the peculiar activity of the central portion of the macronuclear chromatin. A core of chromatin forms in the center of the nucleus, becomes very finely granular, and loses, to some extent, its affinity for basic dyes. A clear zone surrounds it, separating it from the shell of unchanged reticular chromatin (Fig. 2, *B*). At this time the activity of the micronucleus is evidenced by an increase in size and by dispersal of its chromatin as fine granules.

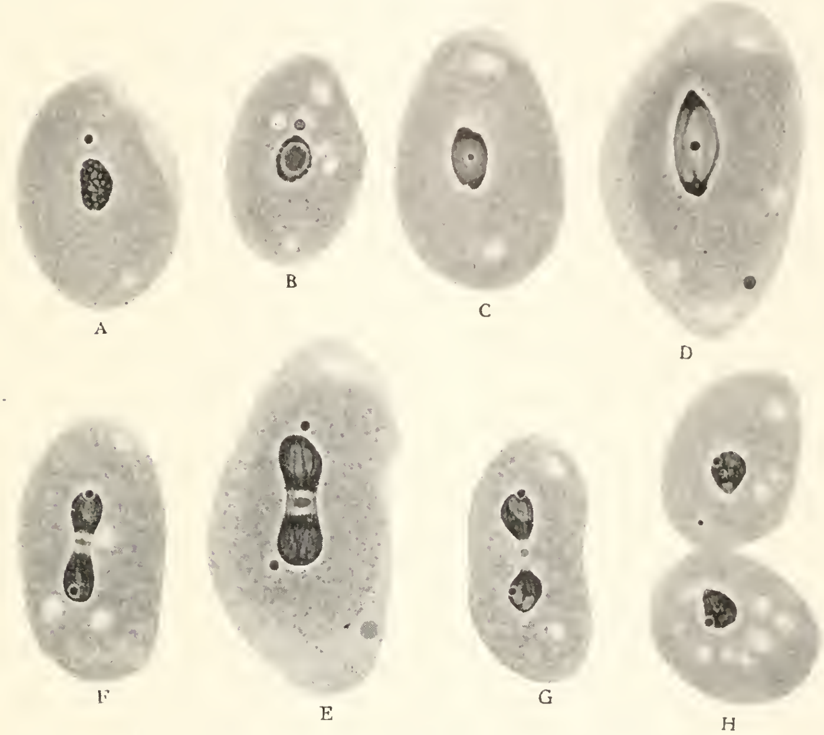

FIG. 2. All figures are of *Allosphærium convexa* during binary fission. The figures represent optical sections of stained organisms drawn with the aid of the camera lucida. × 990.

A. Resting stage. The macronuclear chromatin in the form of a coarse reticulum. Schaudinn-Feulgen.

B. Formation of the central chromatin ball. The shell of reticular chromatin is shown only at the edges in this optical section. Flemming-Heidenhain.

C. Contraction of central ball. Note the concentration of reticular chromatin at poles. Schaudinn-Feulgen.

D. Elongation of macronucleus. Daughter micronuclei below the level of focus. Flemming-Heidenhain.

E. Constriction stage. Most of the reticular chromatin in bands near the division plane. Flemming-Heidenhain.

F. Later stage. The central ball has become loosened and spread out between the future daughter macronuclei. Schaudinn-Feulgen.

G. Separation stage. Schaudinn-Feulgen.

H. Macronuclear division completed. The central ball is cast into the cytoplasm, in this case, of the anterior daughter. Schaudinn-Feulgen.

The central core of the macronucleus gradually contracts and becomes basophilic, ultimately forming a small sphere in the center of a relatively clear region. In the meantime the reticulate shell clumps into large chromatin masses, with the major portion passing to each pole as the nucleus elongates (Fig. 2, *C*). As elongation proceeds the mid-region, with the exception of the central sphere, becomes clear and nearly free from chromatin. Slightly later the chromatin of the reticulum is found massed at the two poles, connected, however, by numerous fine strands that pass around the clear mid-region (Fig. 2, *D*).

As the macronucleus begins to constrict, the entire chromatin content behaves in an unusual manner. Most of the "active" chromatin forms long, thread-like bodies and migrates toward the division plane, leaving, however, two small polar caps. On either side of the division plane the thread-like bodies form two plates, resembling chromosomes in the anaphase (Figs. 2, *E* and *F*). Their appearance is never regular, however, and we would certainly hesitate to call these bodies macronuclear chromosomes. The chromatin of the central ball loses its compactness and spreads out across the division plane. It is always quite definite and stains clearly after the Feulgen reaction as well as with the basic dyes (Fig. 2, *F*). Further constriction results in a second contraction of this "waste" chromatin to form the residual ball. The chromosome-like bodies of the daughter macronuclei become very irregular and more or less fusion of these bodies with the chromatin of the polar caps ensues (Fig. 2, *G*). As the daughter macronuclei separate, the residual ball of chromatin is cast into the cytoplasm (Fig. 2, *H*) where it decreases in size and is finally absorbed. The chromatin of the daughter macronuclei gradually assumes the structure of the resting reticulum as reorganization proceeds. Plasmotomy proceeds to completion and the daughter ciliates separate.

It may be well to mention the appearance of the endoplasmic sphere during cell fission. There is apparently no division of the sphere since it may be identified in its original position during all division phases. It does not change appreciably in size or staining capacity. The new endoplasmic sphere of the anterior daughter seems to arise *de novo* just anterior to the plane of fission. It may be seen first as a faintly staining area (after Heidenhain's hæmatoxylin) which gradually contracts into a sphere. This would lead one to believe that the endoplasmic sphere is a metaplastid representing some product of metabolism. At least it does not appear to be a self-perpetuating structure.

Discussion of Division

In the light of a number of recent reports the extrusion or elimination of chromatin or chromatin-like materials from the macronucleus during binary fission is not particularly unusual. This phenomenon occurs in a number of holotrichous ciliates, e.g., *Loxocephalus* (Behrend, 1916); *Eupoterion* (MacLennan and Connell, 1931); *Kidderia (Conchophthirius)* (Kidder, 1933*a*); *Ancistruma* (Kidder, 1933*b*); *Ichthyophthirius* (Haas, 1933); *Conchophthirius* (Kidder, 1934*b*); *Urocentrum*, *Colpidium*, and *Glaucoma* (Kidder and Diller, 1934). One case that shows striking similarities to that of *Allosphærium convexa* is that described by Rossolimo and Jakimowitsch (1929) in the division of *Myxophyllum* (Raabe, 1934*b*). This ciliate was known until recently as *Conchophthirius steenstrupii* and possesses seven macronuclei in the normal vegetative stage. During division each of the macronuclei, in addition to casting out a part of the chromatin substance, is described as undergoing a type of mitosis. Most of the chromatin takes the form of two groups similar to the chromosomes of a metazoan during the anaphase. There are also two polar caps of chromatin. We have found a number of instances in *Allosphærium convexa* which nearly duplicate the condition in *Myxophyllum*. With such figures at hand one is tempted to draw analogies with true mitosis.

Summary

1. Three species of the amphipod family Orchestiidæ were investigated for ectocommensals. These forms are *Talorchestia longicornis* (Say), *Orchestia agilis* Smith, and *Orchestia palustris* Smith. The material was collected in the vicinity of Woods Hole, Massachusetts.

2. The stomatous, holotrichous ciliates that live as ectocommensals on the amphipods studied belong to two genera: *Allosphærium* gen nov., of which there are five species, and *Chilodonella* Strand, of which there are three new species.

3. All species of ciliates are well adapted to their environment, being small and flat and possessing ventrally placed thigmotactic cilia.

4. The binary fission of one species (*Allosphærium convexa* gen. nov., sp. nov.) is described. A ball of chomatin is differentiated in the macronucleus and extruded into the cytoplasm just prior to cell fission. Most of the macronuclear chromatin takes the form of long chromosome-like bodies during division, duplicating to a fair degree the condition found in *Myxophyllum*.

LITERATURE CITED

Behrend, Kurt, 1916. Zur Conjugation von Loxocephalus. *Arch. f. Protist.*, **37**: 1.

Calkins, Gary N., 1933. The Biology of the Protozoa. Lea and Febiger, Philadelphia.

Haas, Georg, 1933. Beiträge zur Kenntnis der Cytologie von Ichthyophthirius multifiliis Fouq. *Arch. f. Protist.*, **81**: 88.

Kahl, A., 1931. Die Tierwelt Deutschlands. 21 Teil: Protozoa. Fischer, Jena.

Kidder, George W., 1933*a*. Studies on Conchophthirius mytili De Morgan. I. Morphology and division. *Arch. f. Protist.*, **79**: 1.

Kidder, George W., 1933*b*. On the Genus Ancistruma Strand (Ancistrum Maupas). I. The structure and division of A. mytili Quenn. and A. isseli Kahl. *Biol. Bull.*, **64**: 1.

Kidder, George W., 1934*a*. Studies on the Ciliates from Fresh Water Mussels. I. The structure and neuromotor system of Conchophthirius anodontæ Stein, C. curtus Engl., and C. magna sp. nov. *Biol. Bull.*, **66**: 69.

Kidder, George W., 1934*b*. Studies on the Ciliates from Fresh Water Mussels. II. The nuclei of Conchophthirius anodontæ Stein, C. curtus Engl., and C. magna Kidder, during binary fission. *Biol. Bull.*, **66**: 286.

Kidder, George W., and William F. Diller, 1934. Observations on the Binary Fission of Four Species of Common Free-living Ciliates, with Special Reference to the Macronuclear Chromatin. *Biol. Bull.*, **67**: 201.

MacDougall, M. S., 1925. Cytological Observations on Gymnostomatous Ciliates, with a Description of the Maturation Phenomena in Diploid and Tetraploid Forms of Chilodon uncinatus. *Quart. Jour. Mic. Sci.*, **69**: 361.

MacLennan, R. F., and F. H. Connell, 1931. The Morphology of Eupoterion pernix, gen. nov., sp. nov. A holotrichous Ciliate from the intestine of Acmæa persona Eschscholtz. *Univ. Calif. Publ. Zool.*, **36**: 141.

Penard, E., 1922. Études sur les Infusoires D'Eau Douce. Genève.

Raabe, Z., 1934*a*. Über einige an den Kiemen von Mytilus edulis L. und Macoma balthica (L.) parasitierende Ciliaten-Arten. *Ann. Musei Zool. Polonici*, **10**: 289.

Raabe, Z., 1934*b*. Weitere Untersuchungen an einigen Arten des Genus Conchophthirus Stein. *Mem. Acad. Polonaise Sci. et Lettres. Série B: Sciences Naturelles*, p. 221.

Rossolimo, L. L., and Frau K. Jakimowitsch, 1929. Die Kernteilung bei Conchophthirius steenstrupii St. *Zool. Anzeig.*, **84**: 323.

Roux, J., 1901. Faune infusorienne des eaux stagnantes des environs de Genève. *Mem. Instit. Nat. Genevois*, **19**: 1.

Summers, F. M., 1935. The Division and Reorganization of the Macronuclei of Aspidisca lynceus Muller, Diophrys appendiculata Stein, and Stylonychia pustulata Ehrenberg. *Arch. f. Protist.*, (In press).

HYDROGEN-ION CONCENTRATION AND THE RHYTHMIC ACTIVITY OF THE NERVE CELLS IN THE GANGLION OF THE LIMULUS HEART

IPING CHAO

(*From the Marine Biological Laboratory, Woods Hole, and the Department of Physiology, University of Chicago*)

The influence of the hydrogen-ion concentration on the rhythmic activity of the nerve cells in the respiratory center of the vertebrates has been much discussed (Gesell, 1925). Another form of rhythmic activity of nerve cells is represented by the regular nervous impulse originating in the ganglion of the *Limulus* heart, and the influence of hydrogen-ion concentration on these nerve cells can be studied with great convenience. In 1906 Carlson made a preliminary study of the action of acids and alkalies on the ganglionic rhythm. He observed that addition of 1 part of a 0.6 N NaOH or KOH to 100 parts of sea water or plasma had a stimulating effect on the rate and strength of the nervous discharge from the ganglion. A main factor in this effect, however, appears to be the removal of the Mg-ion as $Mg(OH)_2$, the precipitation of which is readily seen in such an alkaline medium. Carlson also pointed out the difference between the action of strong bases like KOH and NaOH and weak bases like NH_4OH. No such difference, however, was observed with strong and weak acids, including HCl, H_2SO_4, oxalic, citric, acetic, tartaric, malic and formic acids. All these acids produce a primary increase in amplitude and frequency of the heart beat, followed by cessation of the rhythm (Carlson, 1906). Carbonic acid apparently acts in a different way, for Newman (1906) observed a primary increase in the amplitude of the heart beat, associated with a marked decrease in frequency, when CO_2 was bubbled through the sea water surrounding the ganglion. More recently, Asher and Garrey (1930) have repeated Newman's work; they found that CO_2 often had little effect on the ganglion for some time, then quite suddenly the contractions became seriously impaired. No particular attention was paid to the influence of hydrogen-ion concentration as such in these earlier experiments. Variation of either the undissociated acid or the hydrogen-ion concentration in the medium (or of both) might conceivably be factors in the quantitative differences just referred to. Accordingly it seemed desirable to repeat some of these observations and to study further the relation of the hydrogen-ion concentration to the rhythmic activity of the ganglion in solutions of strong and weak acids and bases.

The experiments to be described were all carried out on the ganglion of the *Limulus* heart. The ganglion was isolated posteriorly, while remaining in connection anteriorly with two segments of the heart musculature. The muscle was kept immersed in sea water and the contractions were recorded graphically. Test solutions were applied to the ganglion alone, and the hydrogen-ion concentrations were determined colorimetrically.

In the study of the influence of the hydrogen-ion concentration, two kinds of effects should be distinguished: (1) the action of the hydrogen-ion in the medium as such, and (2) the secondary effect of other ions or molecules introduced with the acid or base. For the simple effect of the hydrogen-ion concentration as such, both strong mineral acid and base (e.g., HCl and NaOH) and buffer solutions were used. The effects of weak acid and base were also studied to bring out the difference between the penetrating and the non-penetrating substances.

THE ACTION OF ACIDS

The automatic rhythm of the ganglion is not very responsive to changes of hydrogen-ion concentration as such. Addition of HCl to the unbuffered Ringer's solution[1] (Chao, 1933) produces no well-marked change on the ganglion until the acid reaches a more or less toxic concentration of about N/1,000 HCl. At this concentration the rate of the nervous discharge is accelerated considerably; the intensity of the discharge (as measured by amplitude of contraction) may be slightly increased at first but rapidly diminishes; and the rhythm becomes irregular, showing definite symptoms of injury. Experiments with Ringer's solution buffered with primary and secondary Na-phosphate (3 millimols PO_4 per liter) at pH 5.4 also showed no marked effect.

When CO_2 is bubbled through the Ringer's solution or sea water, the reaction of the solution becomes distinctly acid, but the effect on the ganglion is entirely different from that produced by HCl. As described by Newman (1906), the amplitude of the heart-beat may slightly increase at first; later it decreases with decreasing frequency of the nervous discharge. The degree of these changes appears to depend more upon the concentration of the undissociated carbonic acid molecules than upon the acid reaction. Thus, the average rate of the nervous discharge in 10 minutes for two experiments is − 14 per cent at pH 6.2 and −38 per cent at pH 5.7 as compared with the normal rate in sea water (pH 8.2). At pH 5.2 (at which sea water is practically saturated with CO_2) the rhythmic activity is inhibited in about 5 minutes (average of 7 experiments). The same pH (5.2) can be obtained by addition of about 2 millimols HCl to a liter of sea water, but this solution produces a slight increase in rate; the average rate in 10 minutes for two experiments is increased by 10 per cent as compared with the normal rate in sea water.[2] When the pH is decreased to 4.0 by addition of more HCl, the average rate in 10 minutes is increased by 17 per cent (average of 4 experiments). Further addition of HCl (3 millimols per liter sea water) may increase the rate 50 per cent or more, often followed by serious impairment of the rhythmicity. These experiments indicate clearly that the external hydrogen-ion concentration is not the determining factor in the action of the CO_2-saturated solution. The action of this medium must apparently be attributed to the undissociated carbonic acid molecules which are known to penetrate living cells with great readiness.

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[1] The unbuffered Ringer's solution, containing 445 millimols NaCl, 8.9 millimols KCl, and 37 millimols $CaCl_2$ in a liter, is slightly alkaline in reaction like the sea water (pH about 8.2), due to traces of $Ca(OH)_2$ in the $CaCl_2$.

Acetate buffer (10 millimols acetate per liter) dissolved in sea water or Ringer's solution produces the same general effect but appears to be less effective than the CO_2-saturated solutions at the same pH. Thus at pH 5.2, the CO_2-saturated sea water inhibits the rhythm in about 5 minutes on the average, but the acetate-buffered sea water produces only a decrease of 24 per cent of the average rate in 10 minutes (average of two experiments).[3] The concentrations of acetic acid and carbonic acid in the acetate-buffered and CO_2-saturated sea water respectively are, however, not the same; that of carbonic acid being much the higher. The difference in the concentration of the undissociated acid molecules may account for this quantitative difference in action. Further experiments were performed in which different dilutions of acetate buffer were used at approximately the same pH; these showed clearly that the effect on the rhythm increases with increase in the concentration of acetate buffer.

The Action of Alkalies

Experiments with alkali in sea water can be performed within a rather narrow range of pH only, for Mg, which is present in high concentration (up to 53 millimols per liter) is precipitated at $Mg(OH)_2$ near pH 10. Many calcium salts (e.g., carbonate and phosphate) are also very slightly soluble in alkaline solution. Most of the experiments on the action of alkalies were performed by adding the alkali to the simple Ringer's solution which does not contain any Mg-ion.

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[2] Lactic acid acts like HCl in causing a primary increase in rate followed by decline and irregularity of beat, but is less effective.

[3] Acetate-buffered sea water can also inhibit the ganglionic rhythm but at a lower pH; the rhythm is inhibited in about 3 minutes at pH 4.4 (average of 5 experiments).

The pure effect of OH-ions as such can be demonstrated by adding NaOH to the Ringer's solution. No well-marked effect is seen until the concentration of NaOH reaches N/1,000 or more.[4] In a Ringer's solution containing N/1,000 NaOH, there is either no change in frequency and amplitude or a slight decrease in frequency with a slight increase in amplitude. As alkalinity is still further increased, the decrease in frequency becomes more pronounced, the rhythm becomes irregular, and definite symptoms of injury appear.

Similar experiments were performed with NH_4OH to furnish a comparison between strong and weak alkalies. As described by Carlson (1906), addition of 1 part of a 0.6 N NaOH solution to 4,000 parts of sea water or plasma produces a rapid decrease in the intensity of the nervous discharge with a slight acceleration of rate. The same effect is obtained with NH_4OH in the Ringer's solution; with a somewhat higher concentration of NH_4OH (1 millimol per liter) the rhythmic activity is rapidly inhibited, often to complete cessation of rhythm. This inhibition is temporary. If the ganglion is allowed to remain in the solution containing NH_4OH, a gradual recovery follows; both the rate and the amplitude begin to increase and may finally attain a level well above the initial condition. The temporary inhibitory action of NH_4OH resembles the paradox phenomenon shown under certain conditions in the ganglion (Chao, 1934). The effect, however, is not due to the OH-ion, for it cannot be produced simply by adding sufficient NaOH to inhibit the rhythm completely. Nor can it be referred to the action of the NH_4-ion, for NH_4Cl has an entirely different effect on the ganglion. The addition of 5 millimols NH_4Cl in a liter of Ringer's solution or sea water causes a definite increase in the frequency of the nervous discharge[5] (*cf.* also Carlson, 1906). Apparently the peculiar behavior of NH_4OH is to be attributed to the specific action of the undissociated molecules of NH_4OH. These penetrate living cells readily, in contrast to the lack of penetration or slow penetration of strong alkalies in dilute solution.

In general, the results described in these experiments bring out clearly the difference between the respective influences of weak and strong acids and alkalies on the ganglionic rhythm. The characteristic effects of HCl and NaOH on the rhythm are to be referred to simple changes of pH in the external medium; while in the case of weak acids (like acetic and carbonic acids) or weak bases (like NH_4OH) not only are their physiological effects different from those of strong acid and strong base, but their mode of action indicates the presence of special factors, connected apparently with the penetration of the undissociated molecules into the cell interior.[6]

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[4] Ringer's solution buffered with boric acid and NaOH (2.5 millimols borate per liter) at pH 9.4 has no marked effect on the ganglion.

[5] NH_4Cl is slightly hydrolyzed so as to make the solution more acid. However, the slight stimulating action of NH_4Cl is not an effect of the acid reaction, for the solution can be kept alkaline by addition of a small amount of NH_4OH and yet produces the same effect on the ganglion; e.g., the addition of 0.2 millimol NH_4OH together with 5 millimols NH_4Cl to a liter of Ringer's solution (pH 9.2) still increases the rate. The action of NH_4-ion is, in fact, more like that of K-ion (Chao, 1933).

SUMMARY

The automatic rhythmic activity of the nerve cells in the ganglion of the *Limulus* heart is relatively resistant to changes in pH in the external medium as such. Experiments with strong acid and base indicate that no well-marked physiological effect is seen until toxic concentrations are approached. Thus H-ion (N/1,000 HCl) produces a rapid irregular rhythm with decreasing amplitude of contraction followed by cessation of heart-beat, while OH-ion (N/1,000 NaOH) has an inhibitory effect. Weak penetrating acids (e.g., carbonic and acetic acids) inhibit the rhythm with primary increase in intensity of the nervous discharge, while the action of NH_4OH is characterized by a period of temporary inhibition. The contrast is to be related to the characteristic difference in the readiness of penetration of the undissociated molecules and the ions into living cells.

The author wishes to thank Dr. R. S. Lillie for much help and advice.

REFERENCES

ASHER, L., AND W. E. GARREY, 1930. Some Conditions Affecting the Responses of Limulus Heart to Artificial and Natural Stimulation. *Am. Jour. Physiol.*, **94**: 619.

CARLSON, A. J., 1906. On the Chemical Conditions for the Heart Activity, with Special Reference to the Heart of Limulus. *Am. Jour. Physiol.*, **16**: 378.

CHAO, I., 1933. Action of Electrolytes on the Dorsal Median Nerve Cord of the Limulus Heart. *Biol. Bull.*, **64**: 358.

CHAO, I., 1934. Paradox Phenomena in the Cardiac Ganglion of Limulus polyphemus. *Biol. Bull.*, **66**: 102.

GESELL, R., 1925. The Chemical Regulation of Respiration. *Physiol. Rev.*, **5**: 551.

LILLIE, R. S., 1927. The Activation of Starfish Eggs by Acids. *Jour. Gen. Physiol.*, **10**: 703.

NEWMAN, H. H., 1906. On the Respiration of the Heart, with Special Reference to the Heart of Limulus. *Am. Jour. Physiol.*, **15**: 371.

[6] For a general discussion and literature, see Lillie, 1927, pp. 720–722.

PITUITARY-INDUCED SEXUAL REACTIONS IN THE ANURA

ROBERTS RUGH

(*From the Marine Biological Laboratory, Woods Hole, Mass.*)

Since the original work by Wolf (1929), ovulation has been successfully induced by anterior pituitary treatment in sixteen species of Anura and four species of the Urodela (Rugh, 1935). It is the purpose of this paper to present data on anterior pituitary-induced sexual reactions during the summer months. As a result of these observations it is now possible to say that anuran eggs and tadpoles are available at all seasons.

MATERIAL AND METHOD

The material used was the five species of Anura available in the Woods Hole region and quite generally in the eastern part of the United States. The forms were:

1. *Rana clamitans* (Latreille), the green frog. The normal breeding season starts about the first of June and continues to about the middle of August. Forms caught during June and July which still have their eggs may be kept in the cold room for several weeks before inducing ovulation or amplexus. Very few of the green frogs caught after the first of August will still have their eggs. These eggs cannot readily be distinguished from those of *Rana pipiens.* A single female will give more than 2,000 eggs during a single season.

2. *Rana catesbiana* (Shaw), the bullfrog. Females of this species with body length up to 120 mm. may show rudimentary ovaries but the immature eggs lack pigment and cannot be released from their follicles. These immature females will also lack the secondary sexual character of cœlomic cilia. Females with body length in excess of 130 mm. may be suspected of having mature gonads and hence would be susceptible to anterior pituitary-induced ovulation. The normal breeding season is June and July, a single female giving upwards of 5,000 eggs. Females which lay their eggs early in the season will build up another set of eggs by the end of August, making the frogs susceptible to a second induced ovulation during a single year. This may be due in part to the fact that the bullfrog is a ravenous eater, devouring everything from insects and crustacea to small frogs and turtles. Natural ovulation comes just before the most prolific eating period and the recently emptied ovaries immediately start to build up the eggs for the following season.

3. *Rana pipiens* (Schreiber), the leopard frog. The breeding season of this species depends upon the latitude and ranges from the first of April to the middle of May, more than 3,000 eggs being layed by a single female. Possibly because these frogs are not such ravenous eaters as the bullfrogs, a second batch of eggs is not available until the last of August or the first of September.

4. *Rana palustris* (Le Conte), the pickerel frog. The breeding season of this species runs parallel with that of *Rana pipiens* (April 15–May 15) and a single female may lay upwards of 2,000 eggs. While the eggs are the same size as those of *Rana pipiens*, they are bright yellow or brown in color.

5. *Bufo fowleri* (Hinckley), Fowler's toad. This species has been accorded wide distribution probably because it is so easily confused with *B. americanus*. It is found in great and concentrated numbers near the Coast Guard Station on Cuttyhunk Island, Mass. It requires very little water and is sometimes found buried several inches in the hot sand or hopping rapidly through the grass and weeds of the semi-marshy region. The males are distinguished from the females by the black chin. The breeding season is very long, from about the middle of April to the middle of August and occasionally as many as 6,000 or even 8,000 eggs are laid by a single female.

These Anura were collected in their respective habitats and were used within a few days after they were brought to the laboratory. In general the method used for inducing ovulation was similar to that recently described (Rugh, 1934), namely by the injection[1] of fresh anuran anterior pituitary directly into the abdominal cavity. The mammalian extracts used (whole sheep pituitary and antuitrin-S from pregnancy urine) were supplied through the courtesy of the Parke, Davis & Co.

Experimental Data

More than 150 frogs and 200 toads of the various species were used in the experiments tabulated below. With one exception, each tabulation represents an experiment repeated successfully at least four times. That single exception was induced ovulation in *Rana catesbiana* by using antuitrin-S where a single case (the only one tried) reacted favorably. A single male of the same species reacted to 400 rat units of antuitrin-S by attempting amplexus with a female bullfrog. Additional frogs were not available at the time to repeat and confirm these observations.

Attempts were made to induce inter-generic amplexus, using *Bufo fowleri* and the various species of *Rana*. *Rana pipiens* and *Rana palus-*

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[1] The anterior pituitary is not broken up but is injected whole through a large hypodermic, being suspended either in distilled water or 10 per cent alcohol.

tris were not susceptible to sexual stimulation during the summer period but their pituitaries were used to successfully induce sexual reactions in other Anura. It was also found impossible to induce males of *R. clamitans* and *R. catesbiana* to attempt amplexus with any other than their own species. It has been common experience that male frogs show a great deal more discrimination in their sexual reactions than do the male toads.

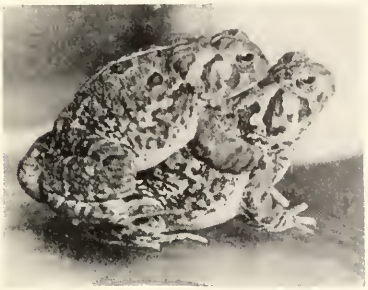
Toads react very quickly to sexual stimulation, and contrary to the situation among the frogs, they are readily susceptible to mammalian extracts of the anterior pituitary hormone. Not only will the female ovulate with antuitrin-S or whole sheep extract but the male will react to these or to any anuran anterior pituitary by attempting amplexus with almost any object that comes within its range.

If a male toad is injected with two male toad anterior pituitaries, and a female with two female pituitaries, amplexus will be induced within 20 hours and often within 4 hours. Such amplexus will be maintained until the eggs are released through the cloaca, and this has been found to be the best method by which to secure developing toad eggs. (Photo 1.) If whole sheep extract (1 cc.) is injected into a male toad and no females are available (either toads or frogs), amplexus will be induced within 9 hours between male and male. (Photo 2.) If a male toad is injected with 0.5–1.00 cc. of antuitrin-S and is placed alone with a female frog (*R. clamitans, R. pipiens,* or *R. palustris*) amplexus will be attempted within 6 hours and often will be successfully maintained for a period of several days. In one case only a male toad attempted amplexus with a female bullfrog, *R. catesbiana*. These cases of inter-generic amplexus always show the male toad in rather an abnormal position. This is to be expected because of the relative sizes of the frog and toad and the very slippery nature of the frog skin. Usually the toad secures a pectoral grip (Photo 3) but occasionally a pelvic grip (Photo 4) is secured and is found to be more firm and permanent.

Inter-generic amplexus has not thus far resulted in successful cross-fertilization. Undoubtedly the male in amplexus emits spermatozoa but numerous unsuccessful attempts at artificial cross-insemination point toward more complex factors which prevent this situation. Thus far the only successful crosses have been between *Rana palustris* eggs and *Rana pipiens* sperm, in which case the tadpoles were kept for thirty days. Further investigation in this direction is in progress.

DISCUSSION

It is quite evident that the sex-stimulating factor of the anterior pituitary is neither sex nor species specific since mammalian extracts can be used to induce either ovulation or amplexus in the Anura. The frogs, and to some extent the toads, usually show convulsive reactions following the injection of these mammalian extracts but this may be due to the phenol preservative, or the tricresol or other substances used in extraction rather than to the anterior pituitary hormone from the mammal. As soon as the male overcomes these convulsive reactions it attempts amplexus. Further investigation should be directed along the line of extraction technique so that the pure and concentrated hormone may be available without any harmful by-products. When such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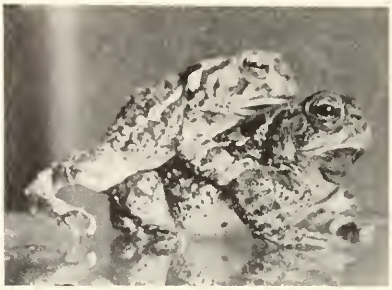
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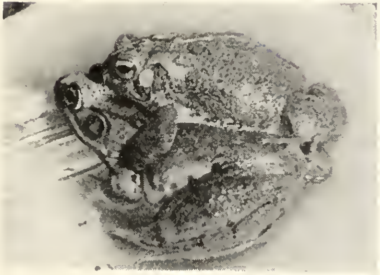
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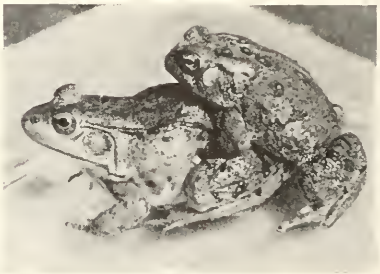
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1. *Bufo fowleri*, male and female in normal amplexus resulting from the injection of standard dose of *B. fowleri* anterior pituitary hormone. Eggs layed in 20 hours, fertilized.

2. Male *B. fowleri*, injected with 1 cc. of extract of whole sheep pituitary shown in amplexus with an uninjected male. Amplexus achieved within 9 hours and maintained for more than 24 hours. Note black chin of ventral male.

3. Pectoral grip of male toad (*B. fowleri*) in amplexus with female green frog (*R. clamitans*) induced by injecting the toad with 1 cc. (100 rat units) of antuitrin-S from pregnancy urine (human). Female not injected. Amplexus maintained for 8–10 hours.

4. Pelvic grip of male toad (*B. fowleri*) in amplexus with female green frog (*R. clamitans*) induced by the injection of antuitrin-S as in Case No. 3. This grip is the more permanent, one pair maintaining amplexus for three days.

(*Note:* These photographs were taken by a Leica Camera with the aid of Mr. E. P. Little of the staff of the Marine Biological Laboratory.)

an extract is available, it is entirely possible that the toad (or frog) may be substituted for the more expensive rabbit in human pregnancy tests.

Anuran anterior pituitaries have been found to be uniformly effective in inducing sexual reactions in any anuran providing, of course, that the injected individual has not just gone through a natural breeding season. In no case has induced sexual response appeared in any way abnormal or has it been accompanied by the convulsive reactions which have been associated with the injection of mammalian extracts of the anterior pituitary.

The dose of anuran anterior pituitary necessary to induce the sexual response is, in general, the injection of twice the amount of the host. However, the pituitaries are not uniformly potent for the various species, nor are the relationship of male to female potencies uniform throughout the group of Anura studied. For this reason the data is given in tabular form. As would be expected, the bullfrog gland is relatively the most potent, but this may be only a quantitative relationship since it is the largest of the anuran glands studied. The toad pituitary was found to be the least potent, a fact correlated with its relative size.

It must be remembered that ovulation induced by the injection of the anterior pituitary hormone is not necessarily an all-or-none reaction. The degree to which the ovaries, in a particular case, are emptied, depends upon the dose of the hormone injected and the time of ovulation depends upon the temperature (Rugh, 1935). Taking advantage of these facts, it is possible to so regulate the dose and temperature that the ovaries may be partially (or completely) emptied at a certain specified time. The fact that three of these species can be used to supply eggs (and tadpoles) during the summer months, and *R. pipiens* and *R. palustris* and many others can be so used during the winter months, makes it possible now to say that embryologists need not be without anuran material at any time.

With *R. clamitans* and *R. catesbiana* fertilized eggs can be secured either by inducing amplexus and allowing the pair to lay the eggs in an aquarium or by artificial insemination (Rugh, 1934). The latter method is to be preferred only because it can be controlled and the exact time of fertilization can be ascertained. If care is taken, one can often achieve 100 per cent fertilization by such means. In the case of the toad, *Bufo fowleri,* the situation is quite different. The eggs are not retained in the uterus as they are in the frogs but are layed single (or double) file as they pass through the cloaca. Thus far it has been impossible to have a female toad discharge its eggs into an aquarium containing concentrated sperm suspension and get the eggs fertilized.

TABLE I

Induced ovulation in Anura during the summer; June 15 to August 15.*

Form	Breeding Season	Source Anterior Pituitary Hormone												Remarks	Time between injection of hormone and appearance of eggs in uterus, 22–25° C.
		Rana clamitans		Rana catesbiana		Rana pipiens		Rana palustris		Bufo fowleri		Antuitrin S	Whole sheep		
		♀	♂	♀	♂	♀	♂	♀	♂	♀	♂				*hours*
Rana clamitans..	June–Aug. 15	2	4	1	1	2	4	2	4	4	6	x	x	Normal fertilized eggs most easily obtained.	17–40
Rana catesbiana.	June–July	4	8			4	8					4 cc. 2 cc. twice daily	x	Produces tremendous numbers of eggs. Single case for mammalian hormone.	40–48
Rana pipiens...	April 1–May 15	x	x	x	x	x	x	x	x	x	x	x	x	Too soon.	
Rana palustris..	April 15–May 15	x	x	x	x	x	x	x	x	x	x	x	x	Too soon.	
Bufo fowleri....	Apr. 15–Aug. 15	2	4	1	1	2	4	2	4	1	2	1.5 cc. .5 cc. twice daily	1 cc.	Most susceptible to induced sexual reactions, both male and female.	7–24

* x denotes failure in reaction.

The only method by which a large percentage of normally fertilized toad eggs can be secured is by inducing amplexus and ovulation simultaneously.

Inter-generic amplexus has been successful only in the one direction illustrated. The toad's skin contains numerous glands, poison to frogs, and this fact alone is adequate to explain the negative results when a male frog is injected in an attempt to induce it to go into amplexus with a female toad. The male toad, however, is very responsive to sexual stimulation and, when injected with the anterior pituitary hormone, will go into amplexus with almost any available object. By amplexus is meant, not merely a temporary gripping by the forelimbs, but the maintenance of the normal amplectic position for at least two hours. In only two out of eighteen cases of inter-generic amplexus has the female frog been found dead, and these cases might not have been due to toad poison. The male toad, in contrast with the male frog, is rather indiscriminate in respect to the selection of an object for amplexus.

TABLE II

Pituitary Induced Amplexus

Form	Source Pituitary Hormone	Reaction Time*
R. clamitans	1 ♀ *R. clamitans* 1 ♀ + 1 ♂ *R. clamitans* 1 ♀ + 2 ♂ *R. clamitans* 2 ♀ *R. pipiens* 3 ♂ *R. pipiens*	No amplexus, inadequate dose Amplexus in 18 hours Amplexus 6–24 hours Amplexus 6–24 hours Amplexus 18–24 hours
R. catesbiana ...	400 rat units antuitrin-S (Parke, Davis & Co.)	Brief amplexus (one case only)
B. fowleri	1 ♂ or 2 ♂ *B. fowleri* 1 ♀ *B. fowleri*	Amplexus in 2–20 hours Amplexus in 2–20 hours
	1 cc. whole sheep extract 0.5–1.0 cc. antuitrin-S	Amplexus 5–9 hours Amplexus 2–6 hours

* Reaction time refers to time between injection of the hormone and amplexus. The duration of amplexus is usually several days or until the eggs are extruded from the uterus. In all cases where *Rana* male is in amplexus with *Rana* female, it has been found that the female was also sexually active. This is not necessary with *Bufo*.

CONCLUSIONS

The doses of anuran anterior pituitary hormone necessary to induce sexual reactions in *R. clamitans*, *R. catesbiana* and *B. fowleri* during the summer months is given.

Methods are described for the securing of fertilized anuran eggs. It is now possible to secure anuran eggs and tadpoles at any season.

Mammalian extracts of the anterior pituitary (whole sheep gland or antuitrin-S from pregnancy urine) are effective in toads but not uniformly effective in inducing sexual reactions in frogs. It is suggested that this may be due to extraction technique, the by-products of which may be actually harmful to the more sensitive frogs. When the technique is further refined it is possible that the Anura may be substituted for rabbits in human pregnancy tests.

The male toad, *Bufo fowleri,* is shown to be indiscriminate when sexually aroused, going into amplexus with male toads or female frogs of various species when female toads are not available. This is not the case with frogs, where discrimination is evident.

LITERATURE CITED

Rugh, Roberts, 1934. Induced Ovulation and Artificial Fertilization in the Frog. *Biol. Bull.*, **66**: 22.

Rugh, Roberts, 1935. Pituitary Relations in Induced Ovulation. *Jour. Exper. Zool.* In press.

Wolf, O. M., 1929. Effect of Daily Transplants of Anterior Lobe of the Pituitary on Reproduction of the Frog (Rana pipiens Shreber). *Anat. Rec.*, **44**: 206.

Wolf, O. M., 1929. Effect of Daily Transplants of Anterior Lobe of Pituitary on Reproduction of Frog (Rana pipiens Shreber). *Proc. Soc. Exper. Biol. and Med.*, **26**: 692.

THE INFLUENCE OF PANTOTHENIC ACID ON GROWTH OF PROTOZOA

ALFRED M. ELLIOTT

(*From the Biological Laboratories, University College, New York University, and the College of the City of New York*)

INTRODUCTION

During the past few years Williams and his associates have been concerned with isolating from many sources substances which stimulate the multiplication of yeast. These investigations finally have resulted in the isolation of a substance to which the name, "pantothenic acid" has been given (Williams et al., 1933). This is an appropriate name because the acid has been found in tissues of representatives of nearly all the plant and animal phyla. It would seem that a substance of such universal occurrence must possess some fundamental biological significance.

Pantothenic acid exerts a pronounced stimulating effect on the growth of the "Gebrüde Mayer" strain of *Saccharomyces cerevisiæ*. Furthermore, it has many properties in common with vitamin G (B_2). Such striking characteristics make it an ideal substance for experimentation with other microörganisms, especially with certain Protozoa because they are more closely related to higher animals than are yeasts. Results of such investigations would be particularly interesting if pantothenic acid should prove to be identical with vitamin G (B_2). Reports in the literature concerning the effects of such substances on the growth of bacteria-free strains of Protozoa are completely lacking, and any evidence to show that Protozoa do or do not require vitamins in their normal nutrition would be of general as well as special interest.

The writer wishes to express his appreciation to Professor R. J. Williams for supplying the pantothenic acid, and also to Miss E. Swaine for counting the organisms in making the tests.

MATERIAL AND METHODS

In testing the effect of such a substance as pantothenic acid on the growth of a protozoan form, it is imperative that the cultures tested are free from other contaminating microörganisms. For that reason all the test cultures used in this investigation are bacteria-free. It was decided to test the effect of pantothenic acid on the growth of a ciliate, *Colpidium striatum*, and a phytomonad flagellate, *Hæmatococcus pluvi-*

alis. The first organism is definitely animal-like in its food requirements while the latter is plant-like in its nutrition. A comparative study was thus conducted with two remotely related protozoan forms, one definitely animal-like, the other plant-like.

The bacteria-free strain of *Colpidium striatum* employed was the one used in a previous investigation (Elliott, 1933). The pure strain of *Hæmatococcus pluvialis* (bacteria-free) was originally obtained from Professor E. G. Pringsheim, and was used recently in a morphological study (Elliott, 1934). Professor R. J. Williams very kindly supplied the pantothenic acid in the form of a calcium salt which had a potency of " 244 "; " one milligram added to 2.5 liters of culture medium gave a heavy response with yeast in 18 hours." Difco tryptone was employed in an organic basic culture medium, which was made up as follows:

Difco tryptone	10.0 gram
KH_2PO_4	2.0 "
Distilled water	1.0 liter

This medium was used throughout the experiments with *C. striatum*, while with *H. pluvialis* the tryptone content was reduced to 0.5 per cent because previous experiments had shown that the flagellate grew better in such a concentration.

For experimental purposes large quantities of the basic medium were made up and subsequently divided into two parts; to one portion was added HCl in titrating for the lower pH values and NaOH was added to the other portion in titrating for the higher pH values. Usually 10 tubes were set at each desired pH unit, each one of which contained 8 cc. of basic medium; to 5 of these (after sterilization) 1.0 cc. of a sterile pantothenic acid solution (10 mg. in 250 cc. of distilled water) was added. To the other 5 tubes distilled water (1 cc.) was added. The tubes were then inoculated with organisms from a stock culture prepared in the following manner. A heavily growing culture tube of the organisms was pipetted into a sterile centrifuge tube and centrifugalized. This was repeated at least four times with sterile tap water. Finally the washed organisms were pipetted into a dilution flask containing 200 cc. of sterile tap water. From this flask 1-cc. inoculations were made into each tube; the flask was vigorously shaken before each inoculation in order to insure an even distribution of the organisms. One tube was then selected from each set and tested for the initial pH. Most of the series were incubated at room temperature (19–26° C.) for a period of 76 hours or longer. After the incubation period was completed, two or more of the tubes from each set were checked for the final pH. One-half cc. of Bouin's fixative was then added to these and the remaining tubes and the final counts made; this was done with the aid of a Sedgwick-Rafter counting chamber and a Whipple ocular micrometer. A LaMotte roulette comparator was employed in making pH determinations.

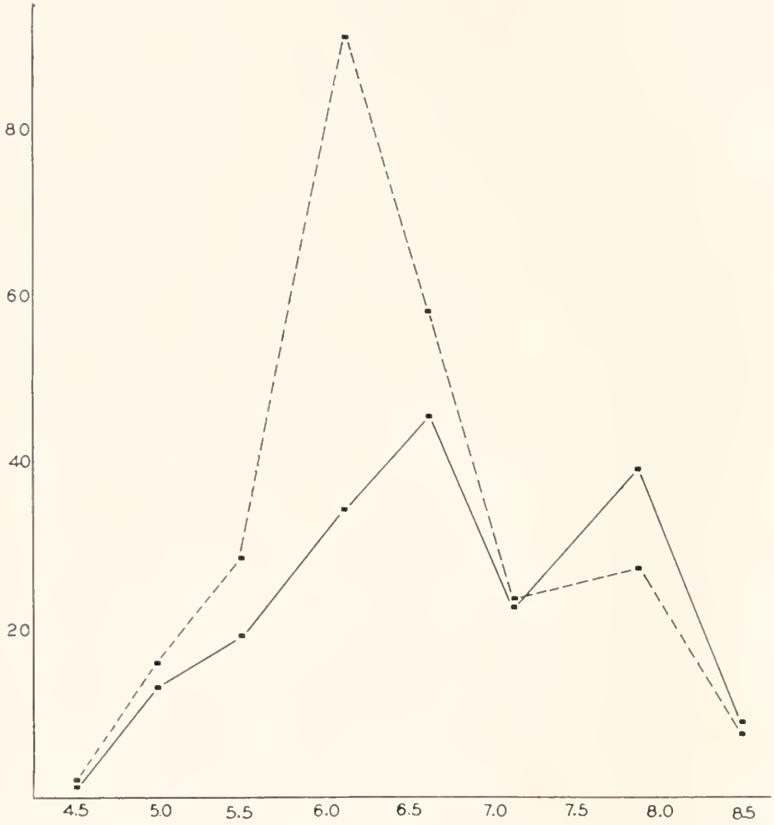

FIG. 1. Series I. Concentration of ciliates in thousands per cubic centimeter plotted against initial pH. The solid line indicates growth in the control; the broken line, growth with pantothenic acid.

EXPERIMENTAL

In testing the effect of any ionizable substance such as pantothenic acid, it is essential to determine its influence over a wide pH range, and not at the point of optimum growth alone of the organism. A substance may have decidedly different effects in the ionized and molecular condition. This fact was well illustrated in the case of acetic and butyric acid (Elliott, 1933a); these two substances were definitely toxic in the lower pH ranges (6.5–4.5), while above pH 7.0 they actually accelerated growth of *Colpidium striatum* and *C. campylum.* For this reason pantothenic acid was tested over a wide pH range.

Series I—Colpidium striatum

The pH values were set at 4.5, 5.0, 5.5, 6.3, 6.6, 7.2, 7.9, and 8.5. After sterilization and inoculation the pH remained the same in all the tubes. After 80 hours of incubation at room temperature there were no pH changes, except in the tubes set at pH 6.3 and there the change was within the experimental error of the readings (± 0.1). The final counts are recorded in Fig. 1.

It is obvious that a marked acceleration occurred in the tubes containing pantothenic acid over those of the controls. A noticeable increase was observed at pH 5.5, with a maximum at 6.3 and 6.6; there was no traceable increase at 7.2 and actual deceleration above this point. Pantothenic acid, as evidenced by this series, accelerates growth of *Colpidium striatum* in the acid range but has no effect in the alkaline range. The bimaximal pH growth curve in the control is typical for this species (Elliott, 1933a).

This entire series was duplicated (Series Ia) with similar results.

Series II—Hæmatococcus pluvialis

The experimental procedure in this series was very similar to that in Series I. The tubes were set at the following pH units: 4.5, 5.0, 5.5, 5.9, 6.5, 7.0, 7.5, 8.2, and 8.5. After 96 hours of incubation at room temperatures and north window illumination there were no pH shifts. The final counts are recorded in Fig. 2.

The results indicate that pantothenic acid had no effect whatever on the growth of *Hæmatococcus pluvialis* over the entire pH range. The differences at pH 7.0 and 8.2 are probably insignificant. It appears then that this substance accelerates the growth of a saprozoic ciliate, yet has no effect on a chlorophyll-bearing flagellate.

Series III—Colpidium striatum

It was shown in a previous investigation (Elliott, 1933b) that gelatin would not support growth of *Colpidium* beyond the third transfer. Gelatin lacks certain amino acids (tryptophane, isoleucine, and hydroxy glutamic acid) but is rich in lysine which is an essential amino acid for the growth of this organism (Hall and Elliott, MSS). Some other substance is apparently lacking in this protein. It occurred to the writer that perhaps pantothenic acid might be such an essential missing substance. For that reason it was thought worth while to determine the effect of pantothenic acid on the growth of *Colpidium striatum* when gelatin was employed as a basic protein. The experimental procedure was very similar in this series to that in the previous two with the exception that Difco gelatin (1 per cent solution) was substituted for Difco tryptone in the basic medium. The pH was adjusted as before at the following values: 4.5, 5.5, 6.5, 7.0, 7.5, 8.0, and 8.5. Pantothenic acid was added to one half of the tubes, sterilized, inoculated, incubated and counted. The final determinations are recorded in Fig. 3.

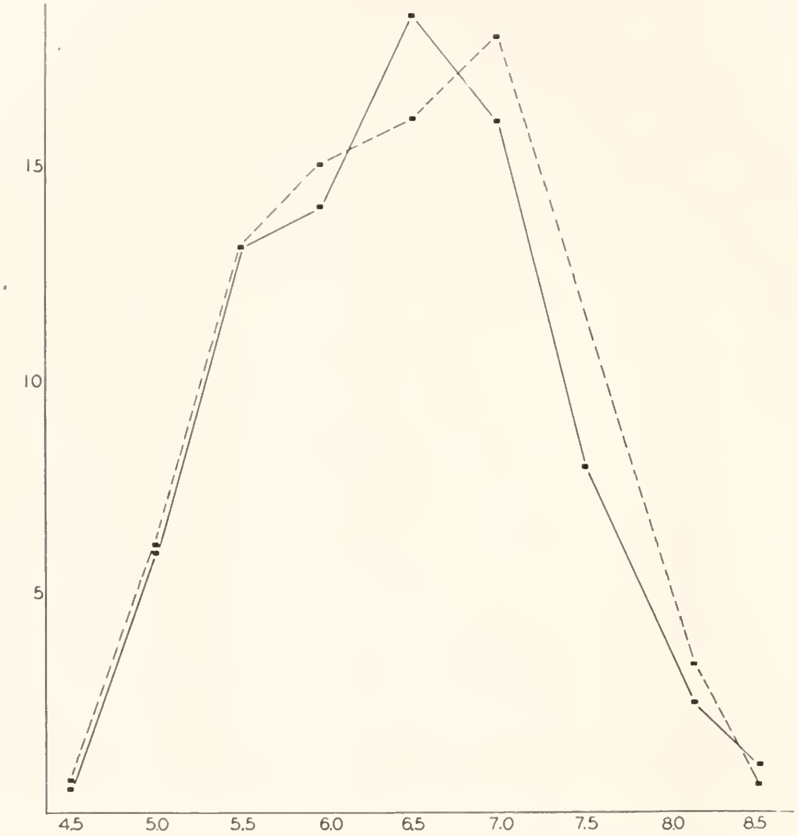

FIG. 2. Series II. Concentration of flagellates in thousands per cubic centimeter plotted against initial pH. The solid line indicates growth in the control; the broken line, growth with pantothenic acid.

The slight growth which occurred in the control tubes was probably due to substances carried over with the original inocula; the stock organisms were not washed as in the previous series. For that reason a small amount of growth would be expected. Had the washings and dilutions been made, no growth would probably have resulted. The essential point, however, is that pantothenic acid is apparently not the substance lacking in gelatin for the support of the growth of *Colpidium.*

Some other, still unknown, substance is deficient in this incomplete protein.

Series IV—Colpidium striatum

It was shown in a previous investigation (Hall and Elliott, MSS) that by adding small quantities of yeast extract to gliadin that growth could be maintained indefinitely; likewise, by adding a small amount of yeast extract to zein, together with lysine which is lacking in this protein, indefinite growth could be maintained. This indicated that some substance in yeast was essential for the growth of *Colpidium*. Perhaps this substance was pantothenic acid? The following series of experiments was devised to test this point.

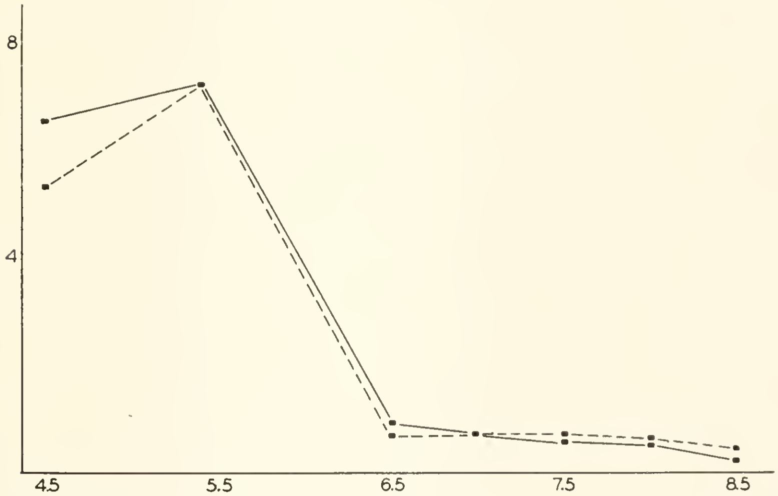

FIG. 3. Series III. Concentration of ciliates in thousands per cubic centimeter plotted against pH. The solid line indicates growth in the gelatin control; the broken line, growth with pantothenic acid.

The procedure was slightly different from that employed in the foregoing series. The basic medium consisted of 1 per cent protein (zein, gliadin, or gelatin) and 0.2 per cent KH_2PO_4 in distilled water. The pH was set at 5.8 in all tubes since previous experiments had demonstrated that acceleration occurred in that range. To 6 tubes containing zein (8 cc. each) lysine was added (1 cc. of 0.7 per cent stock solution); to each of 6 others, was added 1 cc. of pantothenic acid (concentration identical with that used in Series I); and to a third set of 6 tubes was added both lysine and pantothenic acid. In a similar manner lysine and pantothenic acid were added to 3 sets (6 each) of tubes containing gliadin. In this case, however, lysine was added because the concentration of this amino acid in gliadin is low (0.92 per cent). Gelatin was included in this series merely as a check on the previous

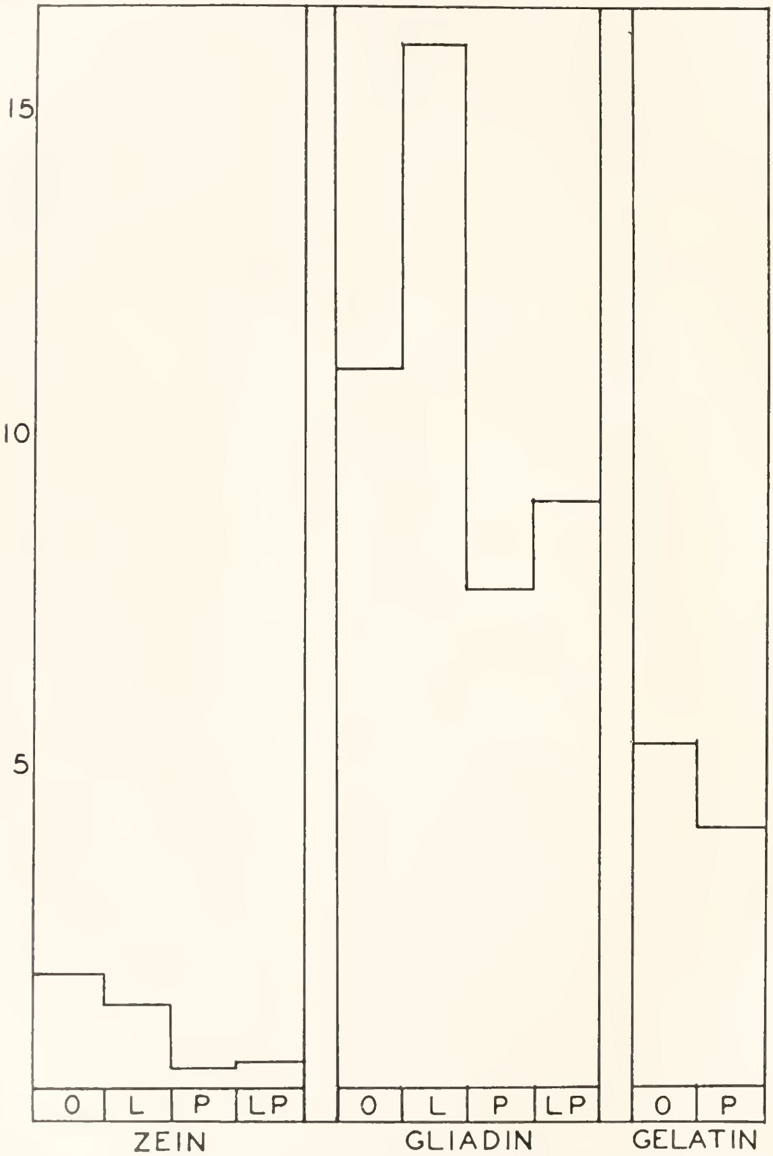

FIG. 4. Series IV. Concentration of ciliates in thousands per cubic centimeter plotted on ordinate. The letters in each column indicate substances added: *O*, nothing; *L*, lysine; *P*, pantothenic acid; *LP*, both lysine and pantothenic acid.

observations. All the tubes were inoculated from a stock culture which had not been washed so that a small amount of the original medium was carried over into each tube. This would permit a small amount of growth even if the tested substance was inadequate to support continued growth. The incubation period was 80 hours and the final counts are recorded in Fig. 4.

It is observed at once that in the case of all three substances, zein, gliadin, and gelatin, pantothenic acid had no accelerating effect. In fact, an actual deceleration is noted in all three cases, although perhaps significant only in the case of gliadin. The growth was comparatively slight in all tubes, which was to be expected if pantothenic acid was ineffective. It may be concluded, therefore, that pantothenic acid is not the substance essential for life of *Colpidium* which is lacking in zein, gliadin, and gelatin.

DISCUSSION

The question of growth-promoting substances in microörganisms has commanded considerable attention for many years. It was initiated by Wildiers (1901), who isolated his so-called "bios" from yeast, and who maintained that this substance was essential for continued growth of the organism. An excellent review of the history of the study of bios is given by Tanner (1925). Fulmer and Nelson (1922) recognized the significant increase of yeast growth by bios but found that it is not necessary for indefinite growth of the cells. Williams (1919) began a long series of experiments which finally resulted in his isolation of pantothenic acid (Williams et al., 1933) which he and his associates claim is identical with, or very closely related to, vitamin G (B_2). Several of its properties suggest such a relationship. It probably is one of the substances originally contained in Wildier's bios. Williams believes that the accelerating substance in the growth of yeast is a single substance, namely, pantothenic acid. Richards (1932), on the other hand, believes that thallium, found as an impurity in asparagin and many other sources, may be the element responsible for acceleration when used in proper concentrations. The striking effect of pantothenic acid on the growth of *Saccharomyces cerevisiæ,* as demonstrated by Williams and his associates (1933), seems more impressive than the results obtained by Richards with thallium. The former workers showed that concentrations as low as 0.02 mg. in 1 cc. of their synthetic culture medium was sufficient to accelerate the growth five times that of the control. Increasing concentrations produced corresponding increases in the growth rate; when the concentration reached 40.0 mg. per cc. of culture medium the fold increase was about 1500 times that of the control. The very high dilutions preclude the possibility of pantothenic acid being utilized as a source of food. Therefore, its activity in the organism probably simulates that of vitamins in higher forms.

Although the effect on Protozoa is not so striking as that produced with yeast, nevertheless the present experiments indicate that the effect of pantothenic acid on the growth of *Colpidium striatum* at certain pH values is very pronounced. The increase is definite on the acid side of neutrality, while above pH 7.0 no acceleration occurred. It was at first thought that this might be due to the toxic effect of the dissociated acid (ionization constant is approximately 3.9×10^{-5} according to Williams and Moser, 1934). However, upon examination of the properties of the acid, it was found that according to Williams it is thermo-labile in the alkaline range. They say, "It is evident that nearly all of its (pantothenic acid) activity . . . is destroyed by prolonged heating in weakly alkaline medium but that heating in weakly acid or neutral medium for 4 hours at 119° C. causes very little destruction." The destruction of its accelerating powers on the alkaline side in Series I might have been due to the heating to 122° C. for 20 minutes when the tubes were sterilized.

The question of synthesis of pantothenic acid by plants and animals was studied by Williams and his associates (Williams et al., 1933). They found that *Aspergillus niger* synthesized the acid in soils and this led them to believe that it was not produced by green plants. They say, "that it is without doubt produced by microörganisms in soil suggests the possibility that it may not be synthesized by green plants." This prediction is of interest in view of the present observations. It was noted that the green phytomonad, *Hæmatococcus pluvialis*, failed to show any acceleration with pantothenic acid while a marked increase was noted with the saprozoöic organism, *Colpidium striatum*. This does not prove that *Hæmatococcus pluvialis* fails to synthesize the acid but it does seem to indicate that the organism does not utilize it to any great extent.

It is not at all unusual that microörganisms are able to synthesize vitamins; for example, Kuroya and Hosaya (1923) showed that *Bacterium coli* synthesized vitamin B, and Damon (1924) demonstrated that several members of the genus *Mycobacterium* produced a growth-stimulating substance analogous to vitamin B. It appears that this ability to synthesize vitamin B is widespread among bacteria and yeasts. Vitamin G (B_2) is probably synthesized along with B since it has somewhat similar distribution and properties. Both vitamin B and G were probably present in the Difco tryptone base (partially hydrolized casein) used in the present experiments, but since all the tubes were autoclaved at one time or another, properties of G were undoubtedly impaired. Furthermore, the writer has maintained bacteria-free cultures of Protozoa for several years in autoclaved media. Since vitamin B is destroyed by such treatment, it is probably not essential for the growth of these organisms. On the other hand, vitamin G (B_2), being heat stable under most conditions, probably was retained unharmed for the most part and possibly utilized by the ciliates. If vitamin G and pantothenic acid prove to be identical substances the results of the present investigation will carry more interest. While these observations do not demonstrate the essential nature of pantothenic acid in regard to continued culturing of *Colpidium siriatum*, they do indicate its importance in rate of multiplication of the ciliate.

Summary

Pantothenic acid, a growth-promoting substance of universal occurrence, was tested for its effect on the growth of two remotely related protozoan forms, namely, *Colpidium striatum* and *Hæmatococcus pluvialis*. The tests were conducted over a wide pH range. In the case of *C. striatum* a doubling of the growth occurred in the pantothenic acid cultures on the acid side of neutrality (pH 5.5–6.6) while no acceleration was observed above pH 7.0. With *Hæmatococcus pluvialis* no differences were noted in the test and the control tubes. It was shown further that pantothenic acid was not the substance lacking in certain incomplete proteins (zein, gliadin, and gelatin) which failed to support indefinite growth of *C. striatum*.

LITERATURE CITED

Damon, S. R., 1924. Acid-fast Bacteria as a Source of Vitamin B. *Jour. Path. and Bact.*, **27**: 163.

Elliott, A. M., 1933a. Isolation of Colpidium striatum Stokes in Bacteria-free Cultures and the Relation of Growth to pH of the Medium. *Biol. Bull.*, **65**: 45.

Elliott, A. M., 1933b. Effects of Certain Organic Acids and Protein Derivatives on Growth of Colpidium. *Anat. Rec.*, **57** (Suppl.): 95. (Abstract.)

Elliott, A. M., 1934. Morphology and Life History of Hæmatococcus pluvialis. *Arch. f. Protist.*, **82**: 250.

Fulmer, E. I., and V. E. Nelson, 1922. Water-soluble B and Bios in Yeast Growth. *Jour. Biol. Chem.*, **51**: 77.

Hall, R. P., and A. M. Elliott, 1934. Growth of Colpidium in Relation to Certain Proteins and Amino Acids. (MSS.)

Kuroya, M., and S. Hosoya, 1923. The Synthesis of the Water-soluble Vitamine by Coli Bacillus Grown on Synthetic Medium. *Sci. Rep. Gov't. Inst. Inf. Dis.*, **2**: 287.

Richards, O. W., 1932. The Stimulation of Yeast Growth by Thallium, a "Bios" Impurity of Asparagine. *Jour. Biol. Chem.*, **96**: 405.

Tanner, F. W., 1925. The "Bios" Question. *Chem. Rev.*, **1**: 397.

Wildiers, E., 1901. Nouvelle substance indespensable au développement de la levûre. *La Cellule,* **18**: 313.

Williams, R. J., 1919. The Vitamine Requirement of Yeast, a Simple Biological Test for Vitamine. *Jour. Biol. Chem.,* **38**: 465.

Williams, R. J., C. M. Lyman, G. H. Goodyear, J. H. Truesdail, and D. Holaday, 1933. "Pantothenic Acid," a Growth Determinant of Universal Biological Occurrence. *Jour. Am. Chem. Soc.,* **55**: 2912.

Williams, R. J., and Robin Moser, 1934. The Approximate Ionization Constant of Pantothenic Acid as Determined by Fractional Electrolysis. *Jour. Am. Chem. Soc.,* **56**: 169.

THE LETHAL ACTION OF SUNLIGHT UPON BACTERIA IN SEA WATER

CLAUDE E. ZOBELL AND GEORGE F. MCEWEN

(From the Scripps Institution of Oceanography, University of California, La Jolla, California)

Although our knowledge of the distribution and activities of marine bacteria is woefully scant, it is recognized that these microörganisms play an important rôle in the biology, geology, and chemistry of the sea (cf. Bavendamm, 1932; Benecke, 1933; and Waksman, 1934). There are several factors which are known to influence the distribution of bacteria in the sea, but so complex is the problem that it is necessary to consider each factor singly in order to evaluate its significance.

It has been stated frequently that the lethal action of sunlight upon bacteria has a profound effect upon their vertical, diurnal, and seasonal distribution. For example, Fischer (1894) found more bacteria in the North Atlantic at sunrise than in the afternoon, and Schmidt-Nielson (1901) attributed to the killing action of sunlight the difference between 26 bacteria per cubic centimeter of surface sea water and 420 per cubic centimeter at a depth of 25 meters. Similarly, Bertel (1912) noted that during May and June, a period of intense insolation, the number of bacteria in the Atlantic between the Azores and Portugal increased from the surface downward and, furthermore, the number of bacteria at the surface increased during the night and was reduced again in the morning. Gaarder and Spärck (1931) ascribed to the bactericidal effect of sunshine the paucity of bacteria in Norwegian oyster pools in the summer as compared to their greater abundance in the winter. Corresponding observations have been made in fresh-water lakes as exemplified by the report of Graham and Young (1934), who found the maximum bacterial population at a depth of 30 to 60 feet, stating that there were fewer bacteria near the surface due to the intensity of the light. Conversely, Reuszer (1933) found no correlation between the numbers of bacteria in surface sea water and the time of exposure to sunshine in the summer months near Cape Cod. Fred, Wilson, and Davenport (1924) found no evidence of light influencing the bacteria in the surface water of Lake Mendota. Lloyd (1930) observed that although the number of bacteria in the surface layers of water in the Clyde Sea area increased slightly during the hours of darkness, even on sunny summer days there were more bacteria at the surface than in the underlying strata, and she deduced that the bactericidal effect of sunlight is negligible. In their studies on the factors which influence the distribution of bacteria in the sea, ZoBell and Feltham (1934) concluded that if there is any direct or indirect harmful effect of sunlight, it is obscured by other factors. This paper is a continuation of those studies.

Experimental

Observations on the vertical distribution of bacteria were made on several bright calm days during the summer months of 1933 and 1934 at the end of the Scripps Institution pier which extends 1,000 feet into the sea and beyond the surf zone. The sampling device consisted of a sterile evacuated bottle fitted with a sealed capillary tube which is broken by a messenger, thus permitting the bottle to fill with uncontaminated water from any level within a few centimeters of the desired depth. For a complete description of the sampling device and details concerning the methods of investigation, evaluation of the experimental error, and the lack of uniformity in the distribution of bacteria in the sea, see ZoBell and Feltham (1934).

Table I

Average plate counts expressed as bacteria per cubic centimeter of sea water collected at various depths.

Depth	Date of experiment		
	6/5/1934	7/2/1934	8/3/1934
meters			
Surface	87	206	48
0.1	62	249	78
0.5	38	164	35
1.0	80	91	73
2.5	94	134	42
5.0	63	282	27

Samples consisting of 50 cc. of sea water were collected at 2:00 P.M. from the surface and at various depths below the surface. After vigorously shaking the samples, appropriate dilutions thereof were plated in duplicate on nutrient sea-water agar. The plates were incubated for four days at 25° C., and the resulting colonies were enumerated in a Stewart counting chamber, using a 3.5x engraver's lens. The average number of bacteria per cubic centimeter are shown in Table I.

This experimental procedure presented no evidence that sunlight destroyed the bacteria in the upper strata of water for, within the limits of sampling errors, there were just as many bacteria in the surface water as in that taken from a depth of 5 meters. These results corroborate previous findings when the mean of twelve samples collected from August 1 to August 13, 1932, was 584 bacteria per cubic centimeter of surface sea water, 447 in the 3-meter strata, and 608 from a depth of 6 meters. It is recognized that the vertical circulation of the water near shore may have a tendency to mix the different strata, but that mixing was not appreciable during this period is indicated by a temperature difference of from 2° to 6° C. between the surface and bottom water at the end of the pier.

The vertical distribution of bacteria to greater depths was investigated near mid-day during the summer months. On the boat " Scripps " bacteriological samples were obtained from intermediate depths to 500 meters at stations ten to fourteen miles offshore where the water was approximately 1,000 meters deep. The results of duplicate analyses of these samples are shown in Table II. The bacterial population was found to increase from the surface downwards to a depth of about 50 meters, which is in accordance with the observations of Føyn and Gran (1928), Schmidt-Nielson (1901), and Bertel (1912).

Table II

Vertical distribution of bacteria at sea expressed as the number of bacteria per cubic centimeter of water at different depths.

Depth	Station no. and date			
	1. 9/7	1. 9/14	3. 6/24	3. 7/16
meters				
Surface	147	344	27	165
5	126	400	47	164
10	238	324	22	253
20	292	528	—	406
50	86	620	109	285
100	14	17	31	98
150	2	0	—	—
200	3	2	2	0
300	6	0	5	2
400	0	1	3	—
500	2	0	2	3

It should be noted that from the surface to the zone of their greatest abundance the number of bacteria increases only two- to four-fold, which is not indicative of a direct bactericidal effect of solar radiations in the upper strata since the penetration of these rays decreases geometrically with depth. If we assume for the sake of argument that the lethal action of sunlight is the only factor which influences the otherwise uniform vertical distribution of bacteria throughout the zone of photosynthesis (which is known to be a false assumption), we would expect the number of bacteria to increase in geometric progression from the surface downward following several hours of exposure to intense sunlight. There is no semblance of such a relationship. As is shown in Fig. 2, virtually all of the bactericidal radiations, or those having a wave length of 3130 Å or less, are absorbed by the first three meters of sea water and there is no significant increase in the bacterial population until a depth of ten to twenty meters is reached. While there is no indication from the study of the vertical distribution of bacteria in sea water that sunlight has any lethal action, the experiments fail to prove that there is no such action because there are multifarious other factors which influence the vertical distribution of bacteria about which little or nothing is known.

Table III

Average plate counts expressed as bacteria per cubic centimeter of surface sea water collected at different times during the day.

Date of collections	Time of collection		
	7:00 A.M.	12:00 M.	5:00 P.M.
June 4	192	167	80
5	180	158	167
6	109	81	102
7	122	—	216
8	204	239	94
11	51	12	34
12	—	103	143
13	217	141	166
14	165	315	72
15	96	283	158
Average	148	167	123

Diurnal and Seasonal Fluctuation

During a two-week period from June 4 to June 16, 1934, 50-cc. samples of sea water were collected from the end of the pier at 7:00 A.M., 12:00 M., and 5:00 P.M. These samples were analyzed for their viable bacteria content by the same standard procedure described above. Table III presents the findings.

While in some cases there was a perceptible diminution in the number of bacteria per unit of water after being exposed all day to sunlight, on other days the 5:00 P.M. samples actually contained more viable bacteria than the early morning samples. The tidal phase was taken into consideration, and it did not influence the results. It is recognized that the same mass of water was not being sampled throughout the day, due to its slow horizontal circulation which accounts for many of the apparent discrepancies. This, together with the fact that bacteria are not uniformly distributed in sea water and that there are certain unavoidable experimental errors in the collection and analysis of samples, emphasizes that the formation of conclusions from one day's observations, as has been done by other workers, is unreliable and untenable. However, most of the errors of single observations should be offset by averaging several observations, and the application of this method revealed that the bacterial counts of the 5:00 P.M. samples were only slightly lower than the 7:00 A.M. samples and, indeed, the 12:00 M. samples contained more bacteria than the early morning ones. Previously, from August 15 to September 13, 1932, by use of a similar procedure, an average for 28 days showed 487 bacteria per cubic centimeter of surface sea water in the early morning and 455 in the late afternoon.

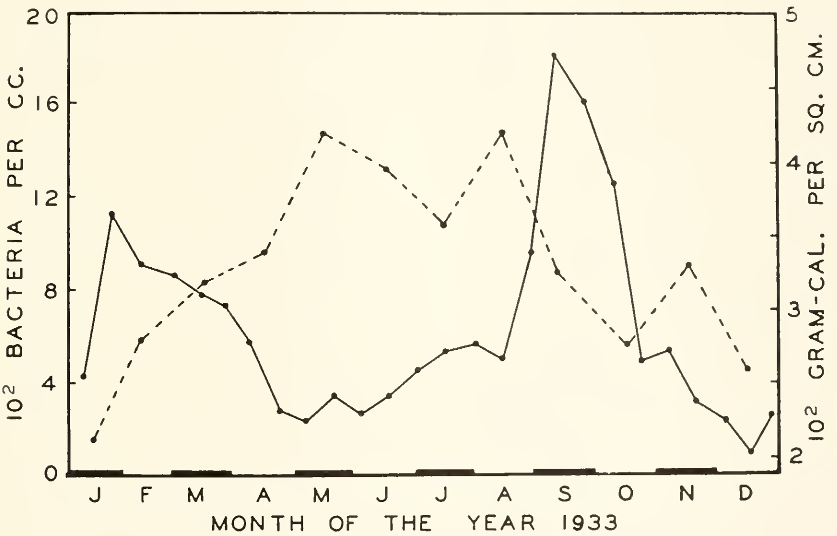

FIG. 1. The solid line gives the bi-weekly average number of bacteria per cubic centimeter of surface sea water during the year 1933 and the broken line gives the corresponding monthly mean solar radiation intensity in terms of gram-calories per square centimeter.

The seasonal distribution of bacteria in the sea has been followed for three years by the daily (except Sunday) analysis of water samples collected from the Institution pier. Data, showing the relationship be-

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tween the bacterial population expressed as bacteria per cubic centimeter of surface sea water and the insolation intensity expressed in terms of gram-calories per square centimeter, are summarized in Fig. 1. Biweekly averages of the former for the year 1933, and the corresponding monthly mean insolation intensity calculated from the daily values recorded by the Institution's pyroheliometer, are given. A description of this instrument appears in the *Monthly Weather Review*, vol. 60: pp. 26–28, 1932, and information on the measurements of solar radiations at La Jolla may be found in subsequent issues.

In Fig. 1 it will be noted that the period of maximum insolation, May to July, was also a period of minimum bacterial population, which from these data alone may be interpreted as a bactericidal effect of sunlight. But it should also be noted that by far the largest number of bacteria occurred in September when the insolation was still relatively intense, and very few bacteria were found the last of November to the first of January when insolation was at its lowest ebb. The same general trends were found in 1932.

Like the foregoing experiments, there are too many additional physical, chemical, and biological factors which also undergo seasonal cycles and which are known to affect the bacterial population of the sea to warrant an evaluation of the direct influence of sunlight alone. For example, it has been found that, except for the large numbers of bacteria which occur each year in February, there is a semblance of correlation between the water temperature and the bacterial population (cf. ZoBell and Feltham, 1934). Also, the phytoplankton content of the water at the same place was highest from May to July, and next highest in November and December, which, if only one factor is considered, may indicate that periods of maximum photosynthesis are accompanied by small bacterial populations and followed by large ones. Thus, while a comparison of the insolation intensity with the seasonal distribution of bacteria indicates that the former may exert a harmful influence on bacteria, the study illustrates the fallacy and futility of trying to interpret findings with insufficient data on contributing factors. Evidence is being rapidly accumulated by field workers that light acts as an important factor in the distribution of both plant and animal species in the sea and the abundance of bacteria is interlinked with the presence of either dead or living organic matter. The vertical distribution of phytoplankton is directly associated with light intensities (Pettersson et al, 1934) and there are numerous phototropic zoöplankton (cf. Spooner, 1933, Gardiner, 1934) which migrate to the surface during hours of darkness and sink to deeper water with increasing luminosity, all of which would affect the vertical, diurnal, and seasonal distribution of bacteria.

CONTROLLED EXPERIMENTS

Direct field observations having failed to answer satisfactorily the question under consideration, laboratory experiments were devised in which the various conditions were under control. During bright days in July when the angle of incidence of the sun's rays approached 90° at mid-day, shallow layers of sea water were exposed in open Petri dishes on the roof of a two-story building. The sea water was paper-filtered to remove suspended particles and thereby insure a more uniform distribution of bacteria. The dishes of sea water were held in a water bath the temperature of which was maintained at 25° to 26° C. with running tap water. Sea water was placed in the dishes having a diameter of 10 cm. to give depths of 2 mm., 5 mm., and 10 mm., this requiring 16 cc., 40 cc., and 80 cc., respectively. The number of bacteria in the water was determined by plate counts at the beginning of the experiment and at intervals thereafter. The average results of three such experiments are summarized in Table IV.

TABLE IV

Average number of bacteria per cubic centimeter of sea water of different depths surviving exposure to direct sunlight from 11:00 A.M. to 1:00 P.M.

Time of exposure	Depth of water		
	2 mm.	5 mm.	10 mm.
Initial	164	159	163
15 minutes	109	135	138
30 minutes	93	124	140
1 hour	83	139	127
1½ hours	81	95	122
2 hours	76	108	126

The experiment clearly demonstrates that sunlight does have a lethal action on bacteria suspended in shallow layers of sea water, this action being most marked during the first few minutes of exposure and being almost negligible after one hour. It also indicates that the bactericidal action apparently decreases with depth even within the upper 10 mm. of water. Such shallow layers of water were also exposed to sunlight from 8:00 A.M. until 4:00 P.M., but the diminution in the number of viable bacteria was not much, if any, greater than when exposed to the mid-day sun for one hour. Nor was there any evidence of a cumulative effect following exposure on five successive days as compared with the controls which were kept in the dark at the same temperature.

In order to examine more closely the relationship of depth to the bactericidal action of sunlight, 38-cm. battery jars were filled with paper-filtered sea water to give a depth of 35 cm. of water. One of these was exposed to bright July sunlight and the other was kept in the dark. Both were maintained at 25° to 26° C. in a bath of running water. Samples were obtained from the surface and at depths of 10, 20, and 35 cm. by means of a sterile pipette, the upper end of which was kept closed until the pipette had been inserted to the desired depth. These samples were collected at 9:00 A.M., 1:00 P.M., 4:00 P.M., and 9:00 P.M., and were plated on nutrient sea water agar. Table V presents the averaged results of three such experiments on three different days.

TABLE V

Average plate counts of sea water in three experiments in battery jars at different depths with and without exposure to sunlight.

Treatment	Depth of sample	Time of taking sample			
		9:00 A.M.	1:00 P.M.	4:00 P.M.	9:00 P.M.
	cm.				
Covered.......	surface	241	202	190	196
Covered.......	10	235	263	188	211
Covered.......	20	228	197	225	208
Covered.......	35	217	204	217	242
Exposed.......	surface	238	152	121	130
Exposed......	10	246	173	198	164
Exposed......	20	209	195	176	153
Exposed......	35	254	211	187	228

Just prior to taking the 9:00 A.M. samples the water in both jars was thoroughly mixed by pouring from one jar into the other, so the difference in the plate counts in the 9:00 A.M. column furnishes an index to the range of error in the analytical procedure. Again it is demonstrated that sunlight kills bacteria in sea water, but it does so to a detectable degree only in the uppermost few centimeters of water, and even in the upper layers the lethal action is relatively slight. From the 4:00 P.M. column in Table V it should be observed that following seven hours exposure to direct sunlight the bacteria in the surface layer were reduced only 36 per cent as compared to the covered control. The average of the four strata at 4:00 P.M. was 205 bacteria in the covered jar and 171 in the exposed jar, and just about the same at 9:00 P.M. Exposure of the water in battery jars on several successive days did not cause any more tangible decrease in the bacterial population than in the unexposed control. In both jars sedimentation and thigmotropism became evident. It has been pointed out by Prescott and Winslow (1931) that the tendency of bacteria to settle in standing water has been misinterpreted as a lethal action of sunlight, and ZoBell and Allen (1933) have showed that many bacteria are thigmotropic and, when confined in glass vessels, attach themselves to solid surfaces.

Although the foregoing results are not always clear-cut, the findings are in perfect accord with our knowledge of the abiotic radiations of sunshine. Recent work by Hulburt (1928), Atkins (1932), Richardson (1932), and others shows that the penetration of light in sea water decreases rapidly as the wave length decreases. This is illustrated by Fig. 2, which gives the depth of penetration of certain bactericidal radiations in terms of the percentage of the incident rays which reaches the stated depth. The data were calculated by using the approved formula and the absorption coefficients found by Hulburt (1928).

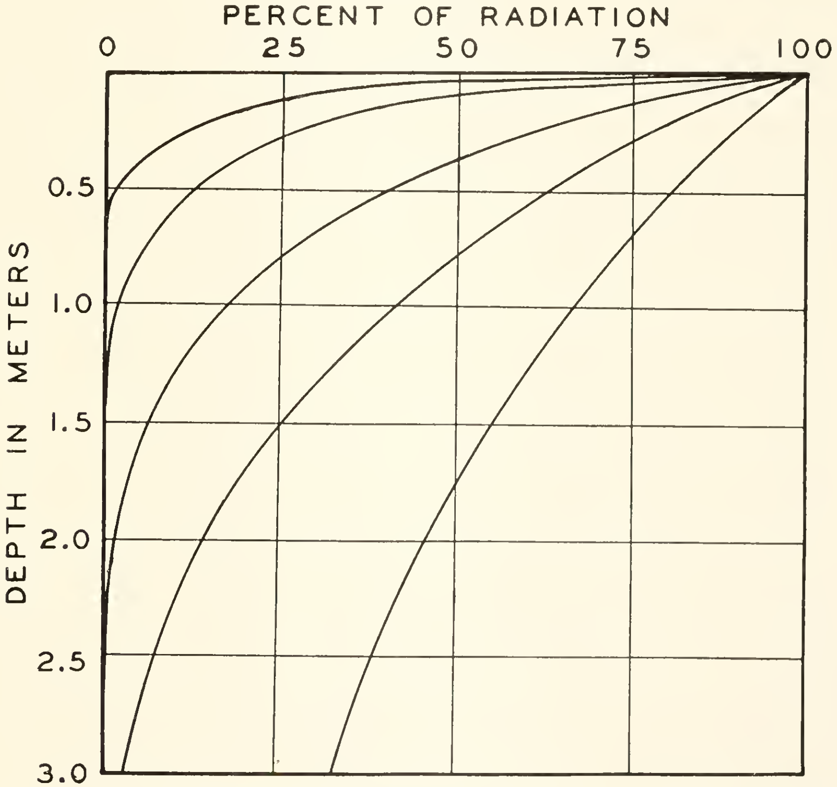

FIG. 2. Percentage of incident intensity of radiations of wave lengths, reading from top to bottom, 2540, 2660, 2800, 3030, and 3340 Å, which penetrate clear sea water to different depths.

From a comprehensive review of the literature, Ellis and Wells (1925) conclude that the bactericidal range of solar radiations is from 2960 Å to 2100 Å, with the maximum between 2800 Å and 2500 Å. This is also the consensus of opinion of the workers whose findings are reviewed by Buchanan and Fulmer (1930). A few investigators claim that wave lengths up to 3660 Å are perceptibly abiotic but, indeed, they are only feebly so, as indicated by the work of Cernovodeanu and Henri (1910), who noted that whereas 3 to 5 hours were required to sterilize a thin emulsion of *Bacterium coli* with wave lengths greater than 3050 Å, it was sterilized in 15 to 20 seconds with shorter wave lengths. Now it will be observed from Fig. 2 that the penetrating power of the radiations of maximum bactericidal action (2800 Å to 2540 Å) is very small, virtually all of these radiations being absorbed by the first meter of sea water and their intensity reduced nearly one-half by passage through only 10 cm. of sea water. The bactericidal action of ultra-violet radiations decreases much more rapidly than the decrease in the intensity, as has been shown by Coblentz and Fulton (1924). Fischer and Holden (1927) found that the lethal action of ultra-violet radiations is determined directly by their intensity and it seems to be a logarithmic function. Furthermore, it should be stated that the data in Fig. 2 are based upon observations in which the angle of incidence of the radiations is 90° and in quiet clear sea water free of suspended matter. The transmission of radiations is reduced proportionately as the angle of incidence decreases and, likewise, when the surface of the water is ruffled by wind or wave action. Also, the penetration is materially less in sea water containing organic matter, especially if the latter is particulate. Grimm and Weldert (1911) found that as few as 100 bacteria per cubic centimeter greatly increased the absorption of ultra-violet rays. Working at sea, Richardson (1932) observed that 21 per cent of the incident rays of sunlight up to 4800 Å is absorbed by the first 5 mm. of sea water, and it will be recalled that these longer wave lengths are far more penetrating than the ultra-violet rays.

Incidentally, it may be of interest to point out that the absorption coefficients of ultra-violet radiations in sea water are much greater than in fresh water. For example, the absorption coefficient as found by Hulburt (1928) for the wave length of 3030 Å is 0.017 in sea water and only 0.005 in distilled water, which means that the incident intensity of this radiation will be reduced to 18 per cent after passing through 10 cm. of sea water, whereas 95 per cent of it will penetrate this depth of distilled water. In natural fresh water Buchner (1893) noted only a feeble lethal action of sunlight on bacteria, the bactericidal power penetrating less than 3 meters, and Jordan (1900) found that in river water the sun's rays are virtually without action. Topley and Wilson (1928) conclude that even in clear water it is doubtful if abiotic rays are active for a distance of more than 5 feet from the surface and, due to the turbidity and constant movement of water in nature it is improbable if bacteria are subjected to the influence of the rays for sufficient time to kill them.

CONCLUSIONS

Observations on the vertical, diurnal, and seasonal distribution of bacteria in the sea fail to show evidence of a lethal action of sunlight. While the seasonal fluctuation in the bacterial population is more or less inversely proportional to the intensity of sunlight, it is recognized that there are multifarious interlinked biological, physical, and chemical factors which also influence the bacterial population of sea water.

Controlled laboratory experiments reveal that at this latitude sunlight has a feeble lethal action on bacteria in the uppermost few millimeters of sea water, but even shallow layers of sea water are not sterilized by prolonged exposure.

Virtually no bactericidal radiations penetrate sea water three meters, and the intensity is materially reduced by passage through 10 cm. of sea water.

APPENDIX

While the data summarized in Table V indicate that sunlight does kill bacteria in sea water, there is an unavoidable residual error or variability of the plate counts even when conditions are held as constant as possible. Therefore, in order to determine details regarding quantitative relations, and to estimate their statistical significance, the following method of analysis was selected as being appropriate:

Assume the variability of the counts with respect to time to be independent of the depth. Determine the statistical significance of differences between the variabilities at different depths using, as is customary, the standard deviation, or S.D., for an index of the variability. Attribute to changes in the controlling factor, radiation, any differences that could not reasonably arise as a result of the sampling or residual error. In order to eliminate the effect of differences in the initial counts of the three experiments, all results of each experiment were expressed in the percentage of the average count at all levels for that experiment at the initial time, 9:00 A.M. The average results, including the S.D. but not the individual counts, are presented in Table VI. Lethal action of sunlight is indicated by the regular decrease of the S.D. of values exposed. On account of the small number or size of sample in each column, tests of significance should be made in accordance with theories of small samples, for example, that of Fisher (1928). There are twelve individual observations per column of individual counts corresponding to eleven "degrees of freedom," and, according to Fisher's Table VI, the natural logarithm of the ratio of any two S.D.'s must exceed 0.52 for chances of less than 5 in 100, and exceed 0.75 for chances of less than 1 in 100. Accordingly, comparing the S.D., 25.6 of surface values exposed with the S.D., 14.5 of bottom values, the chances are about 4 in 100. Comparisons of any two other values of the S.D. corresponding to the exposed jar indicates chances exceeding 5 in 100. Comparisons between the S.D., 25.6, of surface values exposed with the S.D., 10.2, of surface values covered, gives a chance less than 1 in 100. The difference is not significant at the 10-cm. level or the bottom, but at the 20-cm. level the difference between 16.4 and 8.7 corresponds to a chance of 2 in 100.

TABLE VI

Average plate counts of sea water in three experiments in battery jars at different depths with and without exposure to sunlight expressed in per cent.

Time of taking sample	Covered					Exposed				
	Depth in cm.				Average of rows	Depth in cm.				Average of rows
9:00 A.M.	105	105	100	90	100	109	108	89	94	100
1:00 P.M.	88	119	88	109	101	66	77	83	89	79
4:00 P.M.	81	78	96	91	88	55	82	80	80	74
9:00 P.M.	84	90	90	105	92	54	71	65	96	71
Average of columns	90	98	93	99		71	85	79	90	
S.D. of one observation	10.2	20.2	8.7	13.5		25.6	21.5	16.4	14.5	

The evidence points to some influence producing an unexpectedly high variability at the 10-cm. level in the covered jar. Adopting 14, corresponding to the 35-cm. level as the residual S.D. of a single determination, that of the difference between averages of three would be

$$\frac{\sqrt{2}}{\sqrt{3}} 14 = 11.41.$$

Accordingly, a difference of twice this, or 22.8, corresponds to the 5 per cent level and $2.6 \times 11.41 = 29.6$ corresponds to the 1 per cent level of significance, approximately neglecting corrections for sample size. To take into account the effect of sample size, Fisher's Table IV should be used.

LITERATURE CITED

Atkins, W. R. G., 1932. Solar Radiation and Its Transmission Through Air and Water. *Jour. du Conseil*, **7**: 171.

Bavendamm, W., 1932. Die mikrobiologische Kalkfällung in der tropischen See. *Arch. f. Mikrobiol.*, **3**: 205.

Benecke, W., 1933. Bakteriologie des Meeres. *Abderhalden's Handb. der biol. Arbeitsmethoden*, **404**: 717.

Bertel, R., 1912. Sur la distribution quantitative des bactéries planctoniques des côtes de Monaco. *Bull. de l'Inst. Oceangr. Monaco*, No. 224.

Buchanan, R. E., and E. I. Fulmer, 1930. Physiology and Biochemistry of Bacteria. Williams and Wilkins, vol. 2, pp. 156–191, 1930.

Buchner, H., 1893. Ueber den Einfluss des Lichtes auf Bakterien und über die Selbstreinigung der Flüsse. *Arch. f. Hyg.*, **17**: 179.

Cernovodeanu, M., et V. Henri, 1910. Comparaison des actions photochimiques et abiotiques des rayons ultraviolets. *Compt. rend. Acad. Sci.*, **150**: 549.

Coblentz, W. W., and H. R. Fulton, 1924. A Radiometric Investigation of the Germicidal Action of Ultra-violet Radiation. *Sci. Papers Bur. Standards, U. S. Dept. Comm.*, No. 495.

Duclaux, J., and P. Jeantet, 1925. Transparence des eaux naturelles aux rayons ultraviolets. *Compt. rend. Acad. Sci.*, **181**: 630.

Ellis, C., and A. A. Wells, 1925. The Chemical Action of Ultra-violet Rays. Chem. Cat. Co., New York, pp. 236–269.

Fischer, B., 1894. Die Bakterien des Meeres nach den Untersuchungen der Plankton-Expedition. *Ergebnisse der Plankton-Expedition der Humboldt-stiftung*, **4**: 1.

Fischer, R., and M. Holden, 1927. Cited by Buchanan and Fulmer (1930).

Fisher, R. A., 1928. Statistical Methods for Research Workers. Ed. 2, Oliver and Boyd, London.

Føyn, B., and H. H. Gran, 1928. Über Oxydation von organischen Stoffen im Meerwasser durch Bakterien. *Avhand. Utgitt av det Norske Videnskaps-Akad., Oslo*, No. 3.

Fred, E. B., F. C. Wilson, and A. Davenport, 1924. The Distribution and Significance of Bacteria in Lake Mendota. *Ecology*, **5**: 322.

Gates, F. L., 1930. A Study of the Bactericidal Action of Ultra-violet Light. III. *Jour. Gen. Physiol.*, **14**: 31.

Gaarder, T., and R. Spärck, 1931. Biochemical and Biological Investigations of the Variations in the Productivity of the West Norwegian Oyster Pools. *Rapports et Procès-Verbaux*, **75**: 47.

Gardiner, A. G., 1934. Variations in the Amount of Macroplankton by Day and Night. *Jour. Mar. Biol. Ass.*, **19**: 559.

Graham, V. E., and R. T. Young, 1934. A Bacteriological Study of Flathead Lake, Montana. *Ecology*, **15**: 101.

Grimm, K., and C. Weldert, 1911. Sterilisation von Wasser mittels ultravioletter Strahlen. *Chem. Centbl.*, **82**: 1454.

Hulburt, E. O., 1928. The Penetration of Ultra-violet Light into Pure Water and Sea Water. *Jour. Opt. Soc. Amer.*, **17**: 15.

Jordan, E. O., 1900. Some Observations upon the Bacterial Self-purification of Streams. *Jour. Exper. Med.*, **5**: 271.

Lloyd, B., 1930. Bacteria of the Clyde Sea Area: A Quantitative Investigation. *Jour. Mar. Biol. Ass.*, **16**: 879.

Pettersson, H., H. Höglund, and S. Landberg, 1934. Submarine Daylight and the Photosynthesis of Phytoplankton. *K. Vet.-o. Vitterh. Samh. Handl.*, **4**: 1.

Poole, H. H., and W. R. G. Atkins, 1929. Photo-electric Measurements of Submarine Illumination Throughout the Year. *Jour. Mar. Biol. Ass.*, **16**: 297.

PRESCOTT, S. C., AND C. E. A. WINSLOW, 1931. Elements of Water Bacteriology. Wiley & Sons, New York, Ed. 5, p. 12.

REUSZER, H. W., 1933. Distribution of Bacteria in the Ocean Waters and Muds About Cape Cod. *Biol. Bull.,* **65**: 480.

RICHARDSON, B., 1932. Photoelectric Measurements of the Penetration of Light in Sea Water. *Trans. Am. Geophys. Union, 13th Ann. Meeting Nat. Res. Council,* p. 225.

SCHMIDT-NIELSEN, S., 1901. Beitrag zur Biologie der marinen Bakterien. *Biol. Centbl.,* **21**: 65.

SHELFORD, V. E., AND F. W. GAIL, 1922. A Study of Light Penetration into Sea Water Made with the Kunz Photo-electric Cell, with Particular Reference to the Distribution of Plants. *Publ. Puget Sound Biol. Sta.,* **3**: 141.

SPOONER, G. M., 1933. Observations on the Reactions of Marine Plankton to Light. *Jour. Mar. Biol. Ass.,* **19**: 385.

TOPLEY, W. W. C., AND G. S. WILSON, 1928. The Principles of Bacteriology and Immunity. Wm. Wood, New York, vol. 2, pp. 1283–1284.

WAKSMAN, S. A., 1934. The Rôle of Bacteria in the Cycle of Life in the Sea. *Sci. Month.,* **38**: 35.

WAKSMAN, S. A., 1934. The Distribution and Conditions of Existence in the Sea. *Ecol. Monographs,* **4**: 523.

ZOBELL, C. E., AND E. C. ALLEN, 1933. Attachment of Marine Bacteria to Submerged Slides. *Proc. Soc. Exper. Biol. and Med.,* **30**: 1409.

ZOBELL, C. E., AND C. B. FELTHAM, 1934. Preliminary Studies on the Distribution and Characteristics of Marine Bacteria. *Scripps Instit. Oceanog., Bull.,* tech. ser., **3**: 279.

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THE CHEMICAL COMPOSITION OF THE CRYSTALLINE STYLE AND OF THE GASTRIC SHIELD: WITH SOME NEW OBSERVATIONS ON THE OCCURRENCE OF THE STYLE OXIDASE

C. BERKELEY

(*From the Pacific Biological Station, Nanaimo, B. C.*)

The Crystalline Style

The chemical nature of the crystalline style in the lamellibranchs has interested observers from quite early times. Nelson (1918) gives an excellent summary of the opinions held on the subject up to the time of the publication of his paper and concludes that the style is " a structure of colloid nature resembling mucin."

The view that the constitution of the material of the style resembles that of mucin seems to have originated with Barrois (1889), who showed in the case of *Cardium* that it behaved like a globulin in respect of its solubility and chemical reactions, but also had the property of yielding a reducing substance on hydrolysis with dilute acid. In the absence of carbohydrate substances capable of yielding a sugar on hydrolysis he concluded that this indicated the presence of mucin or chondrin. This conclusion is quoted in some of the early papers in which the constitution of the style of other lamellibranchs is discussed (e.g., List, 1902, in the case of *Mytilus,* and Yonge, 1926, in that of *Ostrea*), but without presentation of any further evidence. More recently reference to mucin is omitted and the substance of the style is regarded as " a protein of a globulin nature " (Yonge, 1931 and 1932). Previously to undertaking the present work I had myself completely confirmed Barrois' observations in the case of a number of species, but his conclusion did not seem justified in the absence of more complete knowledge of the nature of the reducing substances resulting from the hydrolysis of the style material. Any glucoprotein would, for instance, react in the manner indicated and no case is made out for mucin or chondrin in particular.

A good deal of confusion existed as to the respective meanings of these two terms until the matter was clarified by the work of Levene and his co-workers (1922). While formerly substances were classified as chondrins or mucins largely on the basis of their physiological origin and physical condition, provided they had the chemical properties of proteins coupled with that of yielding a reducing substance on acid hydrolysis, the terms have now a definite chemical significance. In both cases the protein radicle is combined with a complex acid radicle, in that of the chondrins chondroitin sulphuric acid, in that of the mucins mucoitin sulphuric acid. Both of these acids yield on acid hydrolysis glucuronic acid, sulphuric acid, and an acetylated hexosamine, in the former case acetyl-galactosamine, in the latter acetyl-glucosamine. The presence of either mucin or chondrin can, accordingly, be definitely established by the detection of these substances among the products of hydrolysis.

The styles of the following four species of lamellibranch have been examined from this point of view: *Schizothærus nuttalli, Mya arenaria, Ostrea gigas,* and *Saxidomus giganteus.* These four species were selected partly because of their large size and ready availability in sufficient quantity to supply enough material for analysis, and partly because they exemplify two distinct types of crystalline style. Those of the two first-named species are very solid in texture, dissolve only slowly in distilled water, and remain practically intact when the animals are kept under adverse conditions even until death ensues. Those of the two last-named species, on the contrary, are of a much less solid texture, dissolve readily in distilled water, and disappear very rapidly when the animals are kept out of water. In the cases of all three species of "clam" the styles were removed immediately after the animals were dug, dried in a water-oven to constant weight, and stored for analysis. In that of the oyster this procedure could not be followed exactly because it was so frequently found that the style had already begun to soften, or had entirely disappeared, in animals left exposed on the beds by the receding tide. In this case, therefore, the animals were lifted into a large floating perforated tray and left there for some hours until the styles were found fully developed. These were removed immediately after the oyster was taken from the water and stored in alcohol until they could be transferred to the drying oven.

GLUCURONIC ACID

Glucuronic acid is readily detected in solutions containing relatively little other organic matter by its property of yielding furfurol on boiling with hydrochloric acid. In the presence of any of several polyvalent phenolic substances furfurol gives a series of highly colored compounds. To the solution under test an equal volume of concentrated hydrochloric acid is added and a trace of phloroglucin or orcin; on boiling a violet-red color develops with the former reagent, a violet-blue with the latter.

In application to crystalline style material this test gave inconclusive results since charring of the protein darkened the solutions too much for color-reading to be possible. As used quantitatively, this difficulty is overcome by distilling off the furfurol produced, which can be readily recognised in the distillate by allowing a drop to fall upon aniline-acetate test paper with which it gives a bright cherry red coloration. By this means positive results were obtained from the crystalline styles of each of the four species under consideration. A quantitative comparison was therefore made between them by continuing the distillation in each case until no more furfurol was produced, precipitating it from the distillate with phloroglucin, and weighing as phloroglucide. The detailed procedure was that of the method of Tollens and Kröber for the determination of furfurol derived from pentoses and the results in Table I are calculated from the phloroglucide by Kröber's factor (Browne, 1912).

TABLE I

Species	Weight of Material	Furfurol	Furfurol
			per cent
Schizothaerus nutalli	.5758	.007083	1.23
Mya arenaria	.5939	.007134	1.20
Ostrea gigas	.3890	.004343	1.11
Saxidomus giganteus	.4784	.004653	.97

The four species thus approximate to one another in furfurol-yielding capacity, and hence in the glucuronic acid content, of their crystalline styles, but such difference as occurs indicates a consistently lower content in the more soluble styles.

GLUCOSAMINE

The method described by Elson and Morgan (1933) for the determination of glucosamine and chondrosamine was applied qualitatively to all the acid residues from which the furfurol had been distilled in the determinations of glucuronic acid described in the previous paragraph. If mucin or chondrin were present in the crystalline style, it was anticipated that the corresponding amino sugar would remain in the solutions as hydrochloride after boiling with hydrochloric acid. For this reason the method of Elson and Morgan for glucosamine and chondrosamine was employed rather than the modification for the estimation of the acetylated compounds described in their later paper (1934). The solutions were made up to equal volume and 5 cc. of each was neutralised, filtered, and submitted to the test. In each case a positive reaction was obtained while a blank test with the reagents remained entirely colorless.

A comparison was made quantitatively between the style of *Ostrea gigas* and that of *Schizothærus nutalli.* The residual solutions from the furfurol distillations were again used for this purpose. It was necessary in the first place to ensure that all the amine present had been set free from combination. To this end 5 cc. of each of the solutions was evaporated to small volume and the residue taken up with 5 cc. concentrated HCl and heated in a boiling water bath for two hours under a reflux condenser. The resulting solutions were neutralised and filtered, whereby only slightly colored filtrates were obtained, and the depth of color developed in Elson and Morgan's test compared with that from 5 cc. of the original solution after neutralisation and filtration. All the filtrates were made up to like volume before applying the test. In neither case could any alteration in the depth of color be detected as a result of the further heating with HCl. It was concluded, therefore, that the hydrolysis had already reached a maximum in respect of the amine constituent in the residues from the furfurol determinations. Comparison was accordingly made between the solutions without further hydrolysis.

The weights of *Ostrea* and *Schizothærus* material respectively taken for the furfurol determinations were almost exactly in the ratio of 1 : 1.5 (see table), so that volumes of the residual solutions taken in this ratio represented equal weights of material. Accordingly 5 cc. of the solution obtained from *Schizothærus* and 7.5 of that from *Ostrea* were withdrawn, neutralised, filtered, the filtrates made up to equal volume, and tested. Very little difference could be detected in the colors developed on standing, indicating a practical equality in the glucosamine content in the styles of the two species.

Sulphuric and Acetic Acids

A series of experiments parallel to those described in the foregoing paragraph was carried out with the residues from the furfurol distillations for the detection and attempted estimation of sulphuric acid, substituting precipitation with barium chloride in acid solution for the Elson and Morgan test.

All the solutions gave positive reactions, but the amounts of precipitate obtained from 5-cc. samples were very small and, in the case of *Ostrea*, only just detectable. In 25-cc. samples the precipitates were still too small to determine gravimetrically and further hydrolysis led to no perceptible increase in the amounts. Attempts at quantitative determination were therefore abandoned, but rough estimates were made by eye of the relative amounts of precipitate obtained from aliquots of the four solutions representing equal weights of material. These pointed fairly definitely to the presence of more sulphuric acid in the styles of *Schizothærus* and *Mya* than in those of *Saxidomus* and *Ostrea,* the last named being markedly the poorest in that constituent.

No attempt was made to detect the acetic acid radicle in the residues from the furfurol determinations. There is no method by which the trace whose occurrence would be anticipated from the small amount of material taken for analysis could be recognised with certainty in the presence of the relatively large amount of hydrochloric acid.

Discussion

It has thus been shown that the crystalline styles of the four species investigated contain the essential constituents of the acids characteristic of mucin and chondrin. Elson and Morgan's test does not serve to differentiate glucosamine and chondrosamine, the respective amino sugars of these two acids, so that a definite conclusion cannot be drawn as to which of them is present in the style. A great deal more material than could readily be obtained would be necessary for this purpose, but, judging by its solubility and ease of hydrolysis with acid, it is extremely probable that mucin rather than chondrin is involved.

There seem to be some slight differences in the composition of the styles of the four species investigated, those of *Schizothærus* and *Ostrea,* for instance, agreeing very closely in their content of glucosamine, but differing in that of glucuronic and sulphuric acids. This suggests that the material consists of a mixture of mucin and a glucoprotein having glucosamine as its carbohydrate constituent; or, in other words, of a glucosamine glucoprotein a variable part of which is united with mucoitin sulphuric acid to form mucin.

If this be so, the differences in solubility of the styles in various species may be associated with their content of mucin. Yonge (1932) has shown that the readiness with which the style disappears when the living animals are exposed to adverse conditions can be correlated with the presence or absence of a separate style-sac; those species in which this is not present losing their styles more readily, when the secretion of the style material is inhibited, owing to their exposure to the solvent action of the digestive fluid of the stomach and intestine. While this is undoubtedly the case, it does not seem to explain the variation in solubility of the styles of various species when removed from the animal and placed in distilled water. *Schizothærus* affords an instance of an animal with a complete style-sac, *Ostrea* of one with none. In the former case, the style persists after long periods of adverse conditions, in the latter it disappears very rapidly; but, as has been pointed out, the style of *Schizothærus* dissolves very slowly in distilled water and that of *Ostrea* very quickly and the former appears to have a greater amount of its glucoprotein in mucin combination. Chemical composition, therefore, may also be a factor in the solubility of the style material and influence its rate of disappearance in adverse circumstances under the action of the digestive fluid.

THE GASTRIC SHIELD

The only statement I have been able to trace in connection with the chemical composition of the gastric shield is that of Nelson (1918). " From its consistency and action toward common reagents " this author concludes " it is probably in the nature of chondrin." This conclusion is quoted by Yonge (1926) in support of his view that " the gastric shield is not a secretion, but is formed by the fusion of cilia, originally in response to the irritation caused by the head of the style."

Schizothærus nutalli has a large gastric shield readily separable from the stomach wall and it was obtainable in fairly large quantity. It was therefore taken for investigation.

It was found at an early stage that the shield could be boiled with 60 per cent potash without dissolving or undergoing appreciable decomposition, the structure preserving its characteristic shape even after prolonged boiling and standing. This rendered a chondrin-like composition extremely doubtful since all chondroid substances hitherto investigated have been found to hydrolyse on alkaline digestion. Chitin seemed to be the compound having the property of resisting attack by strong alkali most likely to occur in the situation in which the gastric shield is found and the material was accordingly examined from this standpoint. After boiling for some time with 60 per cent potash, it was thoroughly washed and dried. It was then found to give the reaction characteristic of chitin with sulphuric acid and iodine and to dissolve readily in boiling concentrated hydrochloric acid. The solution in hydrochloric acid gave off ammonia on making alkaline and boiling, it had a strong reducing action on Fehling's solution, and gave a crystalline osazone with phenylhydrazine. All these tests pointed to chitin. Finally some of the material was submitted to the treatment described in the earlier part of this paper for identifying the constituents of the crystalline style. On boiling with hydrochloric acid and distilling, no furfurol could be detected in the distillate. The residual solution gave no reaction for sulphuric acid, but the presence of glucosamine was shown by the test of Elson and Morgan. Chitin consists of polymerised acetyl glucosamine. There is thus no doubt that the substance of which the gastric shield is composed is chitin and that it contains no chondrin-like constituent.

Nelson's (1918) observation that the gastric shield gives the biuret test and breaks up in the process under the action of the strong alkali employed is probably to be attributed to an incomplete separation of the adjacent tissues and of the substance of the crystalline style which is always more or less adherent to it.

As bearing on Yonge's (1926) view of the origin of the gastric shield, it is of interest to speculate whether the shield may not be derived from the substance of the style itself. From the chemical standpoint it does not seem impossible that chitin could be formed from mucin by the elimination of glucuronic and sulphuric acids from mucoitin-sulphuric acid and polymerisation of the remaining acetyl glucosamine. The related compositions of mucin and chitin have previously been discussed from this point of view by Matthews (1916), who draws attention to the occurrence in large quantity of mucin in the skin of the lower vertebrates and of chitin in the hard covering of the arthropods and considers that the " facts are of interest in the light of the theory of Gaskell and Patten that the arthropods were the ancestors of the vertebrates."

The Style Oxidase

The occurrence of an oxidase system in the crystalline style of the lamellibranchs has hitherto been recorded in the cases of thirteen species which I have enumerated in a previous paper (Berkeley, 1933). Two more, *Panope genorosa* and *Pholadidea penita*, may now be added to the list. I have recently detected considerable oxidase activity in solutions of the styles of both of these. The oxidase has now been observed in a sufficiently large range of families to render it extremely probable that its occurrence is general throughout the lamellibranchs.

The presence of the oxidase has not, hitherto, been recorded in the style of any species of gastropod. Unlike the case of the lamellibranchs, the style is by no means of general occurrence in the gastropods. Its presence has, in fact, been observed in only a very limited number of genera. Yonge (1932) gives a complete list of these and points out that the style occurs only in such gastropods as are herbivores and which " either by ciliary currents or by a radula, pass a continuous supply of finely divided food to the stomach."

The only large marine species among them which I have been able to obtain in a living condition is *Crepidula fornicata*. This species is common in the oyster beds at Olympia, Washington, whence a number of live specimens were recently obtained. On arrival, some 24 hours after being taken from the beds, none of those opened had any crystalline styles. After being kept in the sea for some days, a large number died, but all the survivors contained styles and supplied sufficient material to enable me to carry out a series of oxidase tests. The results of these were quite definitely positive.

SUMMARY

The crystalline styles of four species of lamellibranch have been examined chemically to determine whether the material is entirely protein in nature or contains mucin or chondrin.

The material yields on acid hydrolysis, glucuronic acid, sulphuric acid and a hexosamine, in addition to protein. It therefore contains all the essential constituents of mucin or chondrin. The solubility and ease of hydrolysis of the material suggest that mucin rather than chondrin is involved.

The composition of the styles is not quantitatively identical in the four species examined, but varies in such a way as to suggest that the less readily soluble styles contain the larger quantity of mucin.

The material of which the gastric shield is composed is found to be chitin.

The oxidase system previously recorded in the styles of a number of lamellibranchs is now shown to occur in two more species. It is also found to be present in that of the gastropod, *Crepidula fornicata.*

REFERENCES

Barrois, T., 1889. Le Stylet Cristallin des Lamellibranches. *Rev. Biol. Nord du France*, **3**, **4**: 124, 161, 263; **5**: 209, 299, 351.

Berkeley, C., 1933. The Oxidase and Dehydrogenase Systems of the Crystalline Style of Mollusca. *Biochem. Jour.*, **27**: 1357.

Browne, C. A., 1912. Handbook of Sugar Analysis. First Edition, p. 450.

Elson, L. A., and W. T. J. Morgan, 1933. A Colorimetric Method for the Determination of Glucosamine and Chondrosamine. *Biochem. Jour.*, **27**: 1824.

Levene, P. A., 1922. Hexosamines, Their Derivatives, and Mucins and Mucoids. *Mons. Rockefeller Inst. for Med. Res.*, No. 18.

List, T., 1902. Die Mytiliden. Fauna und Flora des Golfes von Neapel, 27, p. 274.

Matthews, A. P., 1916. Physiological Chemistry. Second Edition, p. 324.

Morgan, W. T. J., and L. A. Elson, 1934. A Colorimetric Method for the Determination of N-Acetyl-glucosamine and N-Acetyl-chondrosamine. *Biochem. J.*, **28**: 988.

Nelson, T. C., 1918. On the Origin, Nature, and Function of the Crystalline Style of Lamellibranchs. *Jour. Morph.*, **31**: 53.

Yonge, C. M., 1926. Structure and Physiology of the Organs of Feeding and Digestion in Ostrea edulis. *Jour. Mar. Biol. Ass.*, **14**: 295.

Yonge, C. M., 1931. Digestive Processes in Marine Invertebrates and Fishes. *Jour. Con. Perm. Intern. Explor. Mer.*, **6**: 187.

Yonge, C. M., 1932. Notes on Feeding and Digestion in Pterocera and Vermetus. With a Discussion on the Occurrence of the Crystalline Style in the Gastropoda. *Rep. Great Barrier Reef Exped.*, vol. 1, No. 10.

A QUANTITATIVE STUDY OF THE VERTICAL DISTRIBUTION OF THE LARGER ZOÖPLANKTON IN DEEP WATER

BENJAMIN B. LEAVITT

(*From the Woods Hole Oceanographic Institution,*[1] *Woods Hole, Massachusetts*)

It is now generally accepted that all depths of the ocean are inhabited (Murray and Hjort, 1912, Chap. IX), but the variations in the amount of plankton at different depths have not yet been investigated thoroughly. The data herein presented are meagre but so little actually pertinent data have been gathered previously that they are more valuable than their paucity might suggest.

During the summer of 1933, investigation was undertaken of the quantitative vertical distribution of pelagic animals in deep water in the offing of Woods Hole. It is believed that the nets used (stramin, 6 threads per centimeter) are as adequate as any gear yet devised for sampling the invertebrate communities as a whole, including the bathypelagic fishes up to a size of six inches or so in length. Obviously the giant squids and active large fishes are not proper quarry for tow nets. The micro-zoöplankton is not caught in nets of such coarse mesh; very different methods must be applied to the investigation of microscopic forms.

It has been possible to measure the total volumes of all the catches and to identify and count all the Euphausiacea. It is hoped that in the near future the species of other groups of animals may be identified and the various problems of distribution concerning them may be attacked.

Work at sea was carried on aboard the research vessel "Atlantis." The collections were made 100 to 300 miles south and east of Woods Hole. The locations of the stations were as follows: No. 1733 (36° 54′ N. and 68° 15′ W. to 36° 35′ N. and 68° 33′ W.) July 27–28, 1933; 2500 fathoms. No. 1735 (36° 50′ N. and 69° 16′ W. to 36° 50′ N. and 68° 52′ W.) July 28–29, 1933; 2500 fathoms. No. 1737 (39° 29′ N. and 70° 14′ W. to 39° 46′ N. and 70° 09′ W.) July 30–31, 1933; 1200 fathoms. No. 1739 (39° 42′ N. and 67° 04′ W.) August 12–18, 1933; 2009 fathoms. Of these four stations, one (1733) was located in the offshore edge of the Gulf Stream; one (1735) in the axis of the latter; and one (1737) in slope water inshore from it. The August station (1739) was just outside the continental slope well inshore from the edge of the stream.

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[1] Contribution No. 59.

Because of the danger of contamination always present when open nets are used, it has long been appreciated that for the collection of the larger planktonic animals from a known depth nets are needed that can be lowered closed to the desired depths, then opened, towed horizontally the desired period of time, then again closed before being hoisted. It is also desirable to use several nets simultaneously in series on the wire in order to save time and to eliminate changes of location or speed, and other variable conditions. Many attempts have been made to perfect gear of this sort. Chun (1888, 1903), Agassiz (1888), Fowler (1898), Kofoid (1905), Bigelow (1913), Ostenfeld and Jespersen (1924), Kemp, Hardy, and Mackintosh (1929), Harvey (1934), and others have devised and used closing nets of one kind or another. But, for technical reasons, none have yet been generally used, even on major deep sea expeditions; and for that reason, many of the published statements as to the vertical distribution of bathypelagic animals have rested on meagre evidence.

Kofoid (1911) gives a very good resumé of the various causes for the failure of most of the earlier attempts with closing nets. Some of the nets were too complex and delicate for the rough work at sea; some were too expensive for routine use; some were designed to be lowered open; some were to hang from the end of the wire and so could not be used in series; some were useful only for vertical tows, not for horizontal; and some were too small for the capture of the more active forms. Kofoid's own net was considered too small and too expensive for the present investigations.

To send down open tow nets to different depths and, by the differentiation of their contents, to assume that the differences in the catches reflect the differences in bathymetrical ranges of the species obtained is of practical application only in reasonably shallow tows. This method not only involves the necessity of a great many hauls in order to formulate significant conclusions, which in itself is enough to make it impractical in deep water, but the tremendous increases in contamination of deep tows by animals from the upper levels render this method completely valueless there.

The nets and releasing device described in Nansen (1915) would not serve our purposes as they are designed to be lowered open and as only one net can be used on the wire at one time. The nets and releasing device described by Ostenfeld and Jespersen (1924) are modeled after these and, whereas improvements have been made, the entire apparatus is designed for other purposes and is not applicable to the investigation of the quantitative distribution of bathypelagic plankton in the open sea.

The " Discovery " investigators have tried various kinds of plankton nets and have also done some experimentation with releasing devices. The closing mechanism employed with their large nets is elaborate and expensive, and, as they point out (Kemp, Hardy, and Mackintosh, 1929, p. 196), " the results obtained with these large horizontal nets were not always satisfactory."

The releasing and towing mechanism used by Harvey (1934) is not of adequately rugged construction and the principles on which it works prohibit its use with the large strains involved with two-meter nets.

A closing net to be adapted for the collection of zoöplankton at great depths should meet the following requirements: (*a*) suitable for horizontal tows in order that one particular depth may be investigated and that a fair quantity from this depth be obtained; (*b*) suitable for use in series so that data on different depths may be gathered contemporaneously; (*c*) easy to handle; (*d*) inexpensive; (*e*) large enough to catch an appreciable amount of material at depths where life is not abundant and to catch large and active forms such as Euphausiids, decapods, etc.; (*f*) strong; (*g*) adapted to tow at one level, i.e., that at which it is opened.

The development of suitable gear required considerable experiment. It was thought at the outset that one-meter closing nets of the type described by Bigelow (1913) would be satisfactory, and these were used at Stations 1733, 1735, and 1737. While the releasing device (since improved) had a few minor weaknesses which caused some of the hauls to fail, on the whole this net worked well, and there is no reason to believe that most of the hauls made with it are not dependable samples of the faunas living at the depths where obtained. Mechanical difficulties, however, limit the use of this net to small sizes, and the catches obtained with it were so small as to point to the need of larger nets.

A system was therefore devised for the use of a two-meter net, opened and closed by a single draw-string (Fig. 1, *b, a, c*), operating through the mouth from the inside.

When lowered, the net is hung from the primary release (t_1) on the releasing device by an eye (*a*) spliced into the draw-line. The draw-line (*b a c*) is attached to a ring of rope (*c g c*) which passes through two eyes in the canvas belly band surrounding the net and thence through a series of rings fastened on the outside of the latter. While the net is being raised or lowered closed, the front part of the net itself is everted through the mouth to form a cone (Fig. 1, *A* and *C*). When the primary release (t_1) is tripped by the first messenger, the eye (*a*) is

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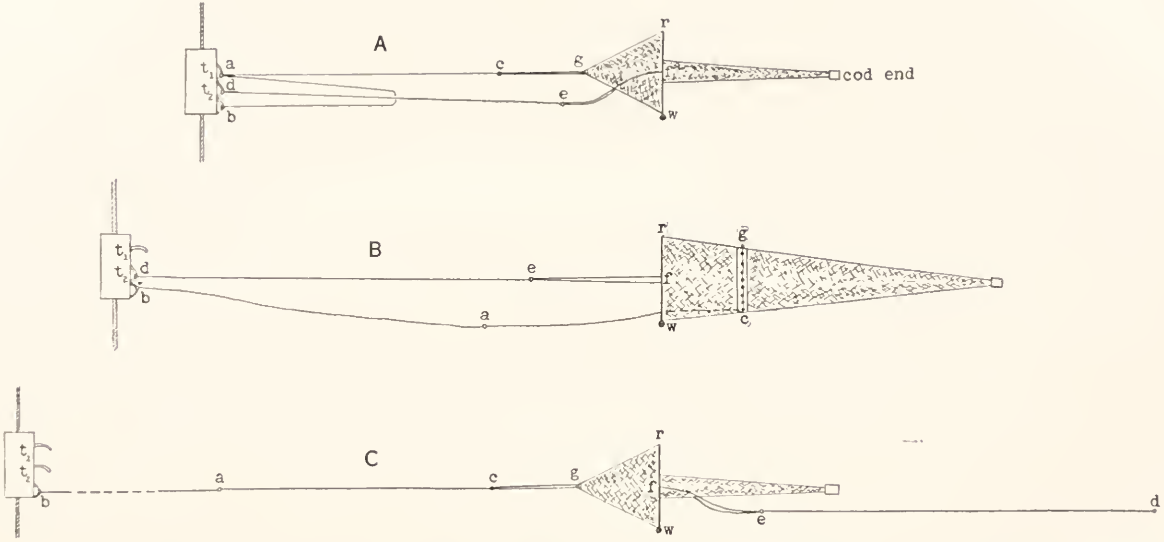

FIG. 1. Closing net used. *A*, net closed for lowering. *B*, net open for towing. *C*, net closed for raising. *b a c*, 9.7 and 6.7-meter draw-string. *c g c*, 7-meter purse line. *d e*, 9.5-meter towing line. *e f*, 3.2-meter towing bridle. *r*, 2-meter diameter rim. *w*, 37-lb. weight. t_1, primary release. t_2, secondary release.

cast off and the force of the water, pouring against the mouth of the net as the ship moves ahead, forces the net open, the pursing line slipping freely through the rings on the outside of the belly band. The pull of the net then comes onto the towing line (*d c*) connected with the bridles (*e f*) (Fig. 1, *B*). While towing, the draw-line (*b a c*) is so slack that the net opens out and operates. After the net has towed open for the allotted time, a second messenger is dispatched which trips the secondary release (t_2), the towing line is thus cast off, and the net comes again onto the draw-line (*b a c*), the end of which is permanently fast to the releasing device. The pull on the draw-line then operates the pursing line around the middle of the net and it is thus closed (Fig. 1, *C*).

When tows with two-meter nets are made from the "Atlantis," it is found most practical to use the ½″ cable with an eight hundred pound weight on the end, to aid in keeping a low wire angle.

After some experiments with the gear, we succeeded in making twenty-nine deep hauls which may be dealt with from a quantitative point of view.

The tows at Station 1739 were all made in an oblique manner through a given stratum of water, by hauling in a prescribed number of meters of wire every five minutes in such a way that the duration of the tow was approximately two hours in most cases. At the other three stations, all tows were horizontal. The best speed at which to tow, keep a reasonably small wire angle, and have the net strain the water well, is about one and one half to two miles an hour. This speed was maintained in so far as possible for all tows, which in turn means that the corrected quantity of plankton caught is roughly that contained in a column of water two meters in diameter and four miles long.

The exact geographic position where each tow began and ended is not recorded here, since it may be assumed that hydrologic conditions were reasonably constant within the region designated by the one determination of latitude and longitude given above for each station. Care was taken not to run far from the given positions, and tows were made in different directions, thus confining them all to an area not too extensive.

The catch from each tow, after the removal of any unusual, large, or delicate specimens, was anaesthetized by the addition of a saturated solution of chloretone and subsequently preserved in dilute formalin. Anaesthetizing plankton before preservation has proven wise in view of the fact that many planktonic animals, particularly the Crustacea, are endowed with the powers of autotomy, and so if placed directly in an irritating preservative, may cast off parts which are of importance in species determination.

In ascertaining the volume of plankton in a given tow, the entire catch is placed on a silk bolting cloth strainer and allowed to drain, but not to dry. It is then added to a known quantity of preservative in a graduated cylinder and the displacement volume of the animals is determined by subtracting the known from the total.

The preliminary sorting and counting were done by the methods used by Mackintosh in dealing with the macro-zoöplankton in the Atlantic sector of the Antarctic (Mackintosh, 1934, pp. 70–72).

As differences in size of mesh of the nets, in speed of towing through the water etc. all cause differences in the amount of plankton caught, it is obvious that uniformity of procedure is essential if the results of different hauls are to be directly comparable one with another. The preliminary hauls here discussed, in which it was necessary to experiment with various nets and with various towing speeds, obviously do not meet the above standard. Therefore, to express the relative abundance I have corrected all tows to the calculated amount that would theoretically have been taken by a net two meters in diameter in two hours towing (Table I, column 8). Even after correction, however, there are still so many unknown factors that the result can only be regarded as a rough estimate of the relative quantity of animals that were living at the time and place where they were caught.

In Table I, the volume of plankton caught is given as measured by displacement, after the salpae have been removed. The removal of the salpae is necessary because their physical properties are such that they do not lend themselves to measurement by the displacement method or any other yet devised. These animals are, however, an important factor in the plankton community and in the metabolism of the sea because at certain times and places there is more material in the form of salpae than in all other types of plankton combined.

The diameter of the net used refers to the diameter across the mouth. The smaller net was scrim, the one-and-one-half-meter net was of silk, and the two-meter nets were of stramin.

Comparison of the quantities of plankton caught at different depths, both by volumes and on a percentage basis, shows clearly that the largest amounts were caught in the upper 300 meters.

At Station 1739 over 93 per cent of the plankton caught was taken between 800 meters and the surface in four out of thirteen hauls. If we subtract the volume of salpae which constituted over 80 per cent of the total catch, approximately 74 per cent was taken between 800 meters

TABLE I

Station no.	Haul no.	Diam. of net used	Duration of tow	Depth	Percentage of total catch at station including salpae	Volume of plankton caught minus salpae	Calc. volume plankton per 2-m. net in 2 hrs. minus salpae
		m.	*hr.*	*m.*	*per cent*	*cc.*	*cc.*
1733	1	1	1	25	55	24	192
"	2	1	1	100	12	5	40
"	3	1	1	300	8	3.5	28
"	5	1	1	300	12	5	40
"	4	1	1	700	13	6	48
					100		
1735	1	1	1	25	29	25	200
"	2	1	1	100	23	20	160
"	3	1	1	300	7	6	48
"	4	1	1	700	8	7	56
"	5	1	2	1200	4	4	16
"	6	1	2	2000	29	25	100
					100		
1737	5	1	1	25	44	120	960
"	4	1	1	100	21	40	320
"	3	1	1	300	28	35	280
"	2	1	1	700	3	35	280
"	6	1	2	1200	.3	5	20
"	1	1	2	2000	3	40	160
					99.3		
1739	1	2	2	400– 0	36	256	256
"	21	2	2	700– 0	14	326	326
"	21	2	2	700– 0	13	146	146
"	19	2	2	800– 500	31	356	356
"	16	2	2	1400–1000	1	18	18
"	17	2	2	1400–1000	1	40	40
"	17	1.5	2	1400–1000	1	6	10.7
"	15	2	2	2000–1600	1	43	43
"	15	2	2	2000–1600	1	57	57
"	13	1.5	1.33	2000–1600	1	15	32.4
"	3	1.5	2	2600–2200	1	85	151.3
"	4	1.5	1.33	3200–2800	2	120	256.3
					103		

and the surface and 26 per cent was taken in depths greater than 800 meters.

The percentages of quantities, including salpae, caught at different depths, at the three deepest stations are as follows:

Station 1735—58 per cent taken above 700 m., deepest haul at 2000 m.
" 1737—93 " " " " 700 " " " " 2000 "
" 1739—94 " " " " 800 " " " " 3200 "

It is true that vertical migrations force us to regard the population of the whole bathymetric province as peculiarly dynamic, particularly so in the photic zone. Nevertheless, it seems sufficiently established that on this particular occasion and at these localities from 75 per cent to over 90 per cent of the animals (by volume) were living in depths less than 800 meters. The works of Chun, Haeckel, Hensen, Fowler, and many others (Steuer 1910, Kapitel V) point to the fact that it is not surprising to find the proportion of animals in the upper eight hundred meters as large as 90 per cent.

TABLE II

Calculated numbers of specimens (Roman type), and numbers per 50 cc. of plankton (italics), per 2-hour haul with 2-meter net at Station 1733.

	25 m.	100 m.	300 m.	300 m.	700 m.
Thysanopoda tricuspidata	8 *2.08*				
Thysanopoda orientalis		16 *20*			
Euphausia tenera			105 *840*		
Euphausia hemigibba	40 *10.40*		9 *72*		
Euphausia mutica	496 *128.96*				
Thysanoessa gregaria			5 *40*	24 *43.2*	38 *304*
Thysanoessa inermis			2 *16*		
Nematoscelis megalops		32 *40*			1 *8*
Nematoscelis atlantica		72 *90*			
Nematoscelis tenella				8 *14.4*	
Stylocheiron carinatum	1824 *474.24*				
Stylocheiron longicorne		248 *310*	27 *216*	48 *86.4*	1 *8*
Stylocheiron elongatum		8 *10*	21 *168*	64 *115.2*	
Nematoscelis sp.		8 *10*			
Young euphausids		48 *60*			

The data also show that there was a minimum quantity of plankton at a depth of 1200 meters, with the quantities increasing progressively both toward greater and toward lesser depths.

This barren stratum is probably what Agassiz (1888) regarded as an azoic zone, and while not actually as poor in animal life as he supposed, the contrast between it and the waters above was striking enough. The explanation of this peculiar distribution is not clear, and whether or not it is a permanent condition fairly universal in its extent in the open sea is an open question, the variation in the abundance even of individual species being often great, both within very short distances, and within very short intervals of time. For example, Michael's (1911) statistics on the numbers of chaetognaths show great differences with successive hauls, both at the same place and in closely proximate places. Single hauls at separate localities can only be regarded as proof that what was caught was present at the particular time and place where the tow was made.

The increase in abundance of plankton in the deep water below the barren zone centering around 1200 meters was thought by Agassiz (1888) to be due to animals gleaning their living from the bottom itself, or to larvae of the bottom fauna which are pelagic during a part of their life histories. This was not the case in the present instance, for at Station 1739, the animals collected at a depth of 3,200 meters (some 600 or 800 meters from the bottom) were a truly pelagic community, i.e. in no way directly dependent on the bottom itself. Thus copepods were abundant throughout the depths, a species of *Metridia* being exceedingly numerous in one haul at a depth of 2,000 meters. Other groups of pelagic animals such as acanthephyridae and other decapods, pelagic ostracods, siphonophores, euphausiids, fish, etc. were represented at all depths from surface down into deep water. Until the species of other groups of animals from these collections have been examined in detail, it would be premature to state just which ones were responsible for the increase in volume of plankton at depths greater than 1,200 meters.

Numbers of Euphausidae

Besides studying the absolute abundance of the total plankton at various depths, it is interesting to learn the relative numerical abundance, one to another, of the individual species in the plankton community. This will clarify two problems concerning the animals caught. First, the relative numbers of animals of the various species at different depths. Second, the number present per unit volume of catch, which tells the proportionate importance of a species or group as compared with the rest of the animals present, i.e. the quantitative rôle which they play as a part of the animal community. In studies of this sort, different investigators have often used such terms as "dominant," "many," "few," "rare," "present," etc. to express quantities; but all such ex-

TABLE III

Calculated numbers of specimens (Roman type), and numbers per 50 cc. of plankton (italics), per 2-hour haul with 2-meter net at Station 1735.

	25 m.	100 m.	300 m.	700 m.	1200 m.	2000 m.
Thysanopoda orientalis			16 *16*			
" *pectinata*			8 *8*			
" *acutifrons*						4 *2*
" *aequalis*		8 *2.48*				
Euphausia tenera	992 *248*	112 *34.72*		16 *14.08*		
" *americana*	128 *32*	8 *2.48*				
" *hemigibba*		152 *47.12*				
" *gibboides*		32 *9.92*				
" *brevis*	384 *96*					
Thysanoessa gregaria						432 *216*
Nematoscelis atlantica				24 *21.12*		
" *tenella*		24 *7.44*	40 *40*			
" *microps*		104 *32.24*	48 *48*	72 *63.36*		
Nematobrachion flexipes			8 *8*			
" *sexspinosus*				8 *7.04*		
Stylocheiron longicorne			224 *224*	8 *7.04*		
" *maximum*				8 *7.04*		
" *insulare*		144 *44.64*				
" *suhmii*		1064 *329.84*				
" *carinatum*	9472 *2368*	96 *29.76*				
" *elongatum*	24 *6*		24 *24*			
" *affine*	8 *2*					
Euphausia sp.				8 *7.04*	28 *86.8*	
Young euphausids	2568 *642*	728 *225.68*		16 *14.08*		

pressions break down in practical use. "Dominant," for instance, is impractical because of the deceptions due to size, color, aggregations, etc., of various kinds of animals. Thus a few *Acanthephyras* may appear to dominate a tow or a single chain of *Salpae* may change the "complexion" of another. We are, therefore, reduced to the necessity of making actual counts for the determination of numbers.

The Euphausiacea are the only group of which total counts have as yet been made. In cases where one or two species which it is impossible to separate from others with the naked eye occur together, it has been found sufficient, for specific enumeration within the group, to separate out three samples of twenty specimens each, from a dish in which the whole catch of euphausiids has been isolated from the rest of the catch of plankton. They were first well stirred and then sampled by taking out the twenty animals lying nearest to a point on the edge of the dish. Identification under the microscope of three such samples of Euphausidae, from Station 1739, revealed *Euphausia americana, E. mutica,* and *E. brevis* in the following proportions:

TABLE IV

Calculated numbers of specimens (Roman type), and numbers per 50 cc. of plankton (italics), per 2-hour haul with 2-meter net at Station 1737.

	25 m.	100 m.	300 m.	700 m.	1200 m.	2000 m.
Euphausia krohnii	16 *.8*	152 *22.80*				
" *gibboides*		8 *1.20*				
" *tenera*			8 *1.44*			
" sp.		8 *1.20*				
Thysanoessa gregaria	136 *6.80*	1544 *231.60*				
" *longicaudata*			104 *18.72*	1000 *180*	148 *370*	948 *293.88*
Nematoscelis megalops	120 *6*	5224 *783.60*	3424 *616.32*			

Euphausia americana	16	15	18
" *mutica*	3	3	2
" *brevis*	1	2	0

As these counts agree so closely, it seemed justifiable to consider this sampling method adequate.

TABLE V

Calculated numbers of specimens (Roman type), and numbers per 50 cc. of plankton (italics), per 2-hour haul with 2-meter net at Station 1739.

	400–0 m.	700–0 m.	700–0 m.	800–500 m.	1400–1000 m.	1400–1000 m.	1400–1000 m.	2000–1600 m.	2000–1600 m.	2000–1600 m.	2600–2200 m.	3200–2800 m.
Thysanopoda aequalis		27 *4.05*										
" *orientalis*	1 *.19*											
" *tricuspidata*			1 *.34*									
" *acutifrons*				1 *.14*								
" sp.	2 *.38*		7 *2.38*									
Euphausia tenera	643 *57.87*	504 *75.60*	604 *205.36*									
" *hemigibba*	12 *2.28*	148 *22.2*	36 *12.24*									
" *pseudogibba*	5 *.95*											
" *krohnii*	462 *79.78*	375 *56.25*	106 *36.04*									
" *americana*	459 *87.21*	70 *10.50*	68 *23.12*									
" *brevis*	25 *4.75*											
" *mutica*	137 *26.03*											
" sp.	2 *.38*			4 *.56*								
Nematoscelis megalops	164 *31.16*	22 *3.30*	11 *3.74*	6 *.84*								
" *microps*	84 *15.96*	21 *3.15*	20 *6.80*	6 *.84*								

TABLE V—*Continued*

	400–0 m.	700–0 m.	700–0 m.	800–500 m.	1400–1000 m.	1400–1000 m.	1400–1000 m.	2000–1600 m.	2000–1600 m.	2000–1600 m.	2600–2200 m.	3200–2800 m.
Nematoscelis tenella		7 *1.05*	6 *2.04*									
Thysanoessa gregaria	12 *2.28*							1 *1.16*	2 *1.76*			
" *parva*				45 *6.3*		84 *105*		20 *23.20*	2 *1.76*	277.55 *416.32*	36 *11.88*	401.4 *76.26*
" *longicaudata*		2 *.3*	63 *21.42*	2 *.28*		19 *23.75*						
" sp.		114 *17.10*			7 *19.6*							
Stylocherion maximum	238 *45.22*											
" *carinatum*		1 *.15*	3 *1.02*									
" *affine*			3 *1.02*									
" *abbreviatum*		15 *2.25*										
" *elongatum*		7 *1.05*	7 *2.38*									
" *longicorne*	1 *.19*											
" sp.		6 *.90*										
Nematobrachion boopis		1 *.15*										
Bentheuphausia ambylops						5 *6.25*	1.78 *8.07*	1 *1.16*	1 *.88*			
Young euphausids			9 *3.06*									

The number of each species of euphausiids has been obtained either by actual count or by the above-mentioned sampling method. To make all catches comparable, the counts of each individual species have then been reduced to a 2-meter net in 2 hours basis. A simple additional calculation of numbers of euphausiids per unit volume (50 cc. of total catch) then shows the relative importance of the former in the latter.

The following example will illustrate this method of calculation; Station 1735; Haul 2; depth 100 m.; total catch 20 cc.; net used 1 m.; duration of tow 1 hr.; cc. per 2-meter net in 2 hrs. 160; number of *Euphausia tenera* actually present 14; number of *E. tenera* in 160 cc. 112; number of *E. tenera* in 50 cc., per 2-hour tow with a 2-meter net, 34.72.

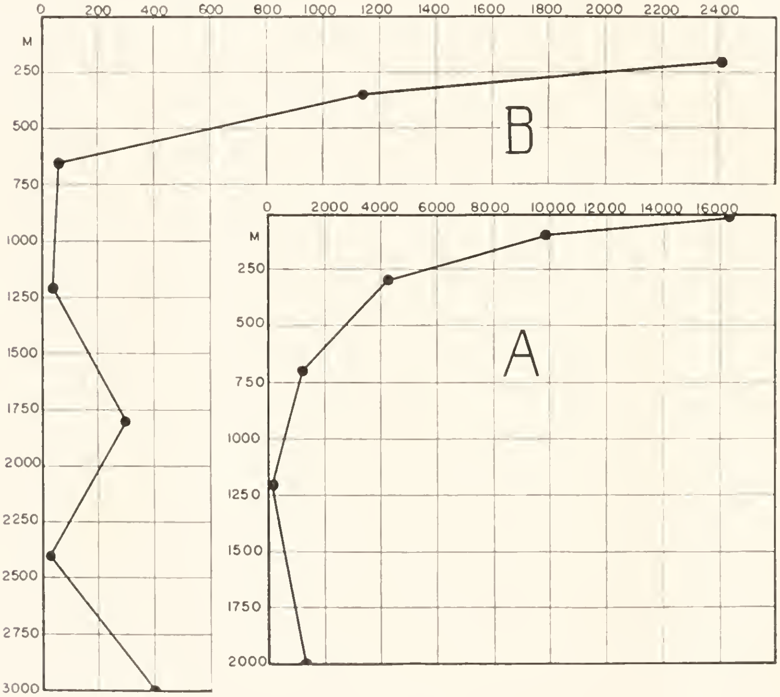

FIG. 2. *A*, Total numbers of euphausiids calculated per 2-meter net per 2-hour haul, Stations 1733, 1735, and 1737. *B*, Total numbers of euphausiids, per 2-meter net per 2-hour haul, Station 1739, determined from average catch at average depths.

Figure 2 shows that the number of euphausiids of all kinds decreased quite regularly with increasing depth at Stations 1733, 1735, and 1737, down to 1,200 meters, with no trace of a secondary maximum at about 700 meters such as appeared in the vertical distribution of plankton vol-times at the time. And while numbers of euphausiids like quantities of plankton increased below 1,200 meters, the importance of this group in the total animal community decreased at these same stations right down to the deepest layer sampled (Fig. 3, *A*), the number of euphausiids per 50 cc. of plankton being much smaller at depths greater than 500 meters than in the upper 300 meters. This vertical relationship also obtained for Station 1739 (Fig. 3, *B*), though at this locality there was a secondary maximum in relative importance of euphausiids at about 1,800 meters.

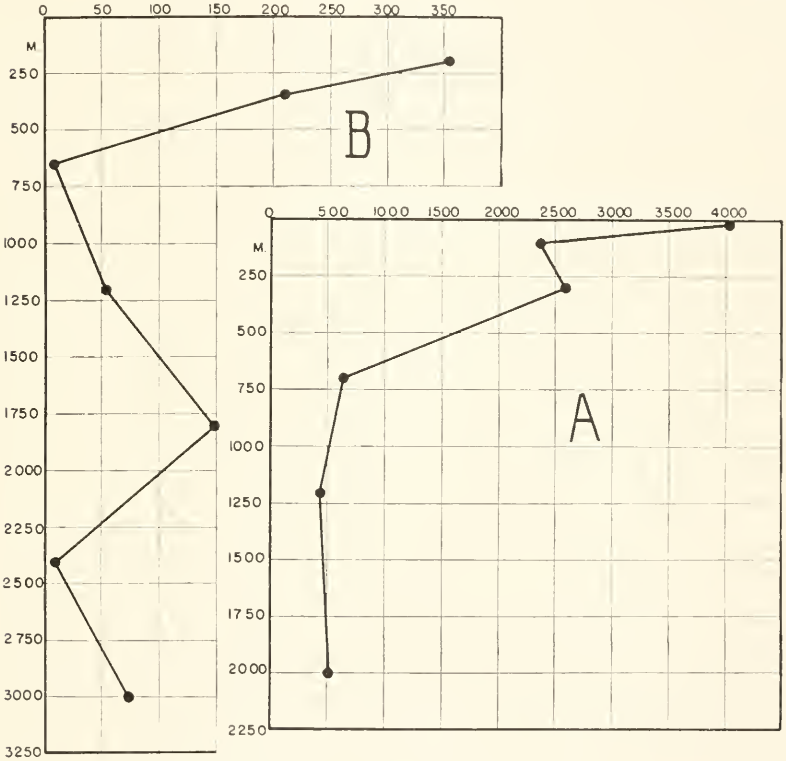

FIG. 3. *A*, Calculated numbers of euphausiids per 50 cc. of plankton from different depths at Stations 1733, 1735, and 1737. *B*, Calculated numbers of euphausiids per 50 cc. of plankton from different depths at Station 1739, determined from average catch at average depths.

This decrease, with increasing depth, both in numbers and relative importance of euphausiids, is furthermore accompanied by a corresponding decrease in the specific diversity of the group, the counts listed in Tables II–V showing that whereas some fifteen species are taken in the upper 400 meters of water, only five (*Thysanoessa longicaudata*, *T. gregaria*, *Bentheuphausia ambylops*, *Euphausia acutifrons*, and *Thysanoessa parva*) were found at depths greater than 800 meters. And of these five, the last two can alone be regarded as truly bathypelagic species on the basis of the present evidence.

Thus it seems sufficiently established at least for this part of the ocean and time of year that euphausiids as a group reach their highest development in the upper water layers.

BIBLIOGRAPHY

Agassiz, A., 1888. Three Cruises of the United States Coast and Geodetic Survey Steamer "Blake" in the Gulf of Mexico, in the Caribbean Sea, and along the Atlantic Coast of the United States, from 1877–1880. *Bull. Mus. Compar. Zool.*, **14**: 1; **15**: 1.

Bigelow, H. B., 1913. A New Closing-net for Horizontal Use, with a Suggested Method of Testing the Catenary in Fast Towing. *Internat. Rev. ges. Hydrol. u. Hydrogr.*, **5**: 576.

Chun, C., 1888. Die pelagische Thierwelt in grosseren Meerestiefen und ihre Beziehungen zu der Oberflachenfauna. *Bibl. Zool.*, **1**: 1.

Chun, C., 1903. Aus dem Tiefen des Weltmeeres Schilderungen von der Deutschen Tiefsee Expedition. Fischer, Jena.

Fowler, G. H., 1898. Contributions to our Knowledge of the Plankton of the Faeroe Channel. No. VI. Description of a new mid-winter tow net. Discussion of the mid-water fauna (misoplankton). Notes on Doliolum tritonis and D. nationalis, and on Parathemisto abyssorum. *Proc. Zool. Soc. London*, for the year 1898, p. 567.

Harvey, H. W., 1934. Measurement of Phytoplankton Population. *Jour. Mar. Biol. Soc. Plymouth*, **19**: 761.

Kemp, S., A. C. Hardy, N. A. Mackintosh, 1929. Discovery Investigations Objects, Equipment and Methods. Discovery Reports, **1**: 143.

Kofoid, C. A., 1905. A Self-closing Water Bucket for Plankton Investigations. *Cons. Perm. Internat. Expl. Mer. Publ.*, Circ. No. 32, p. 1.

Kofoid, C. A., 1911. On a Self-closing Plankton Net for Horizontal Towing. *Univ. Calif. Publ. Zool.*, **8**: 311.

Mackintosh, N. A., 1934. Distribution of the Macroplankton in the Atlantic Sector of the Antarctic. *Discovery Reports*, **9**: 65.

Michael, E. L., 1911. Classification and Vertical Distribution of the Chaetognatha of the San Diego Region. *Univ. Calif. Publ. Zool.*, **8**: 21.

Murray, J., and J. Hjort, 1912. The Depths of the Ocean. MacMillan and Co., Ltd., London.

Nansen, F., 1915. Closing-nets for Vertical Hauls and Horizontal Towing. *Cons. Perm. Internat. Expl. Mer. Publ.*, Circ. No. 67, p. 3.

Ostenfeld, C. H., and P. Jespersen, 1924. Standard Net for Plankton Collections. *Cons. Perm. Internat. Expl. Mer. Publ.*, Circ. No. 84, p. 3.

Steuer, A., 1910. Planktonkunde. Druck und Verlag von B. G. Teubner. Leipzig and Berlin.

OBSERVATIONS ON THE COLOR CHANGES AND ISOLATED SCALE ERYTHROPHORES OF THE SQUIRREL FISH, HOLOCENTRUS ASCENSIONIS (OSBECK)

DIETRICH C. SMITH AND MARGARET T. SMITH

(From the Bermuda Biological Station and the University of Tennessee, Memphis, Tennessee)

On transference from a dark to a light-colored background the common Bermudian squirrel fish, *Holocentrus ascensionis* (Osbeck), shows an exceptionally rapid color change from red to white. This change is coincident with and the result of a pigment concentration within the numerous erythrophores of the scales and the deeper layers of the skin. The speed with which this is effected is strikingly demonstrated when a red fish from a dark background (in these experiments such backgrounds were usually black) is placed in a white aquarium containing three or four animals in the pale condition. In five seconds the originally red fish is indistinguishable from the earlier occupants. Similarly a pale fish placed in the company of red fishes in an aquarium over a black background loses its distinctive coloring in about ten seconds. Such rapid color changes are rarely met with among the fishes, at least under laboratory conditions, although they are not uncommon among the lizards. In the scorpion fish of the Bay of Naples, *Scorpæna ustulata*, a red form comparable in color to *Holocentrus* with erythrophores as the dominating type of pigment cell, the shift from red to the pale condition is a matter of hours (Smith and Smith, 1934). In melanophores the change in distribution of the pigment granules in response to a variation in background from white to black or vice versa is usually completed in three to four minutes. The melanophores of *Fundulus heteroclitus* behave typically in this manner.

The rapidity with which *Holocentrus* is capable of altering the distribution of pigment within its erythrophores is suggestive of a high degree of nervous control over the activities of the chromatophores. Such a supposition is borne out by experiments based upon the methods originally employed by Pouchet (1876), von Frisch (1911, 1912) and others in working out the relationship between the nervous system and chromatophores of fishes. These methods rely principally upon sectioning the sympathetic chain at various levels along its course and noting the effect of such operations upon the subsequent behavior of the pigment cells in the area affected by the operation. In *Holocentrus* if the section involves the anterior half of the sympathetic chain, the area in front of the cut turns red and stays red regardless of background. If the cut, on the other hand, involves the posterior half of the chain the reddened area is confined to the regions behind the cut. In *Holocentrus* severance of the sympathetic then results in a typically persistent expansion of the denervated erythrophores, the distribution of the pigment-motor fibers to these erythrophores conforming to the scheme originally announced by von Frisch (1911) as applying to the innervation of the melanophores of *Phoxinus lævis.* In both species the pigment-motor fibers emerge from the spinal cord at a point about halfway down the trunk and are distributed both anteriorly and posteriorly by way of the sympathetics to the chromatophores in question. Operations of the nature just described demonstrate clearly the existence of sympathetic pigment-motor fibers capable of governing the responses of the erythrophores of *Holocentrus.*

In operated fishes kept upon a white background the resultant red or denervated areas produced by cutting the posterior sympathetic chain were visible in whole or in part for ten or fifteen days after the operation. In such denervated regions the first sign of recovery of function on the part of the affected erythrophores appeared in those cells lying most anteriorly in the operated area. In animals kept upon a white background this portion paled within three or four days after the operation, the paling gradually spreading each day more and more posteriorly until finally the whole area had lost its red color. Such animals when placed on a dark background showed a uniform reddening over the entire body. Repetition of the operation at its original site led to an exact duplication of the original reddening. This reëstablishment of the denervated condition following a second operation together with the nature of the recovery from the original operation is not in disagreement with the assumption that there is a gradual regrowth of nerves into the denervated region together with reëstablishment of nervous connections with the erythrophores. Denervations of the erythrophores of the anterior half of the trunk present a different picture. Here the recovery is much more rapid, so rapid in fact that the possibility of nervous regeneration is excluded. Subsequent operations at the original site often failed to produce the original results. In fact, the general impression received after comparing the behavior of denervated areas in the head and anterior trunk with those in the posterior trunk strongly suggest some essential difference between the innervation of the erythrophores, or else, what seems even less likely, a structural or functional difference between the erythrophores in these two parts of the body. Unfortunately the opportunity was not given us to pursue this matter further. A somewhat similar relationship between dorsal and ventral trunk melanophores in *Phoxinus* has also been reported (Smith, 1931).

Further proof of the nervous control of the erythrophores in *Holocentrus* is obtainable on stimulating the anterior end of the medulla where, following the work of von Frisch (1911), one would expect to find the pigment-motor center. When by means of a suitable stimulating electrode this is done in *Holocentrus* the entire animal immediately pales and stays pale as long as the stimulus is applied. On stopping the stimulation the animal reddens, the erythrophore pigment assuming the state which typically follows the necessary operative procedure involved in exposure of the posterior portion of the brain. The original paling may be reproduced at will as long as the chromatophores remain in a reactive condition. Furthermore stimulation of the opthalmic nerve at a point where it crosses the superior surface of the orbit produces a marked ipsilateral paling of the head (Pouchet, 1876; v. Frisch, 1911; Smith, 1931).

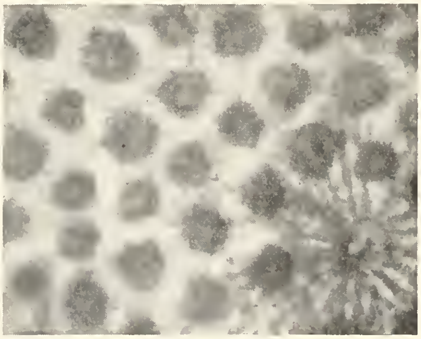

FIG. 1. Pulsating erythrophores in the expanded phase in N/10 NaCl. A non-pulsating expanded melanophore in the lower right-hand corner.

A reversal of the effect of temperature changes on the erythrophores of the trunk before and after sectioning the sympathetic chain gave further evidence of an innervation of these cells. Exposure of the unoperated trunk to a locally directed stream of warm sea water (35° C.) produced in the affected region a pronounced expansion of the erythrophores, as shown by the increase in the red color of the surface of the body at the point warmed. This reddening was strictly confined to the area heated. If, however, the stream were directed upon an area of the trunk in the same fish denervated a few days previously the erythrophores now contracted and the heated area became pale. A similarly directed stream of cold water produced in the innervated trunk a contraction and in the denervated trunk an expansion of the erythrophores. This curious reversal of the responses to heat and cold on the part of certain chromatophores after denervation has previously been noted in connection with the melanophores of *Fundulus heteroclitus* (Smith, 1928). Such reversals are not apparently the rule among fishes as attempts on other occasions to evoke the same response in the chromatophores of other forms (*Phoxinus lævis, Scorpæna ustulata, Trigla* sp.) have been unsuccessful, the direction of the response of the chromatophores of these fishes to temperature changes remaining the same whether denervated or innervated. This was also true with another Bermudian form, the surgeon fish, *Acanthurus hepatus*. The extent to which this peculiarity characteristic of the chromatophores of *Fundulus* and *Holocentrus* is spread among other fishes should be a matter worth investigating further.

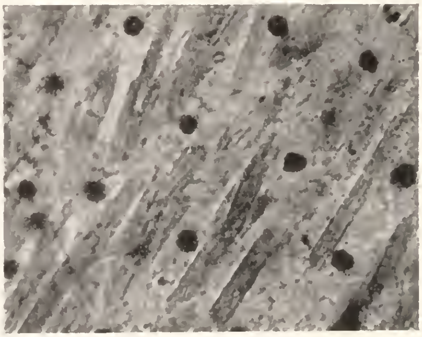

FIG. 2. Pulsating erythrophores in the contracted phase in N/10 NaCl.

The scales of the squirrel fish upon microscopical examination proved to be favorable objects for direct observations upon the activity of the chromatophores. Three types were present, erythrophores, xanthophores, and melanophores, the erythrophores being the most numerous with an even distribution throughout the pigmented area of the scale, while the xanthophores, less abundant than the erythrophores, were largely confined to the distal corners. The melanophores were relatively scarce and were more or less irregularly scattered throughout the scale. In sea water isolated scales showed chromatophores in the semi-expanded condition, that is with only the proximal one-third or less of the processes of the cell filled with pigment. In N/10 NaCl, however, the pigment distribution was no longer a static affair, the erythrophores and the xanthophores instead alternating between states of expansion and contraction, the pigment granules migrating rhythmically in and out of the processes (Figs. 1 and 2). These pulsations first appeared at the proximal border of the pigmented area, occurring almost simultaneously with the color changes of the iridocytes (Smith, 1933). This would indicate that the pulsations were an immediate response to the penetration of the NaCl into the tissues of the scale. The pulsations were relatively slow at first, beginning with two or three a minute, a level from which they gradually increased to between fifteen or twenty-five a minute. At this point the rate either gradually declined until pulsations ceased entirely or else it continued to increase further. In this latter event rates of fifty to sixty a minute were not uncommon, the extent of the pulsations becoming progressively less and less until it was almost impossible to tell whether the cells were pulsating at all, the erythrophores apparently remaining contracted. Usually after the erythrophores had ceased pulsating a period of quiescence set in lasting from three to five minutes, followed by a second and on occasion even a third or fourth period of activity. Thus the time in which the pulsatory activity could be seen usually lasted forty-five minutes to an hour and a half, although now and then individual cells were seen to pulsate for three hours or more, long after all the rest of the erythrophores in the scale had become quiet. During the first few minutes of the pulsations the movements of all the erythrophores as well as the xanthophores were synchronous throughout the scale. As time went on, however, the chromatophores comprising the pigmented area broke up into smaller groups, each group proceeding to pulsate at a rhythm independent of the others. This synchronism might indicate some sort of coördinating mechanism within the scale. The rate and extent of the erythrophore pulsations in one scale, however, compared with another were extremely variable, scarcely any two scales being even approximately alike.

The failure of the melanophores to pulsate under conditions which excite the erythrophores and xanthophores to activity does not indicate that these cells are incapable of such activity. Pulsations may be induced in the melanophores by use of a method originally introduced by Spaeth (1916). This requires a five-minute exposure of the cells to N/10 $BaCl_2$ followed by a transfer of the scale to N/10 NaCl. In *Holocentrus* scales so treated the melanophores show good pulsations in about five minutes after the immersion in NaCl following treatment with $BaCl_2$. These pulsations differ from those seen in the erythrophores and the xanthophores in that they are much slower, occurring only once or twice a minute. Slow pulsations of this sort are also characteristic of the melanophores of *Fundulus heteroclitus* in which such activity has been extensively studied (Spaeth, 1916; Smith, 1930).

Erythrophore pulsations have been reported before. In fact this type of behavior on the part of chromatophores was first observed in connection with the erythrophores of *Mullus*. Ballowitz (1913) described rhythmical movements of the pigment granules in the erythrophores of excised bits of the skin of this form when such pieces of the skin are immersed in 0.75 per cent NaCl. Judging from his descriptions, erythrophore pulsations in this form were of the slow type seen in the melanophores of *Fundulus*. The isolated scale erythrophores of *Scorpæna ustulata* can also be made to pulsate (Smith and Smith, 1934) but only after they have been previously treated with $BaCl_2$. In this respect the *Scorpæna* erythrophores resemble more the melanophores of *Fundulus* and *Holocentrus* than the erythrophores of *Holocentrus*. In *Scorpæna* the erythrophores pulsate steadily for an hour or so at a rate of about once every two minutes. Melanophores in the same scale pulsate somewhat more rapidly, about once a minute on the average.

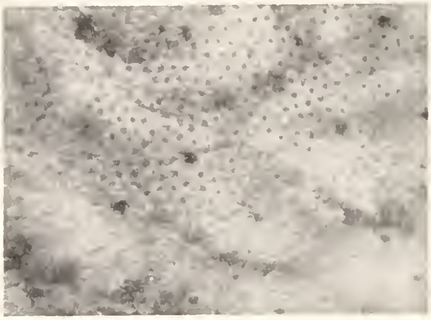

FIG. 3. Showing the progressive contraction of the chromatophores in N/10 KCl after sea water. Cells in the lower right contracted, cells in the upper left still expanded. The small bodies are erythrophores and the larger ones melanophores.

When *Holocentrus* scales are placed in N/10 KCl all of the chromatophores show a marked contraction (Fig. 3). This is quite in keeping with the results of other investigators upon the effect of KCl upon isolated scale chromatophores. In $CaCl_2$ the erythrophores and xanthophores of *Holocentrus* show a brief period of pulsatory activity lasting about eight minutes, after which they assume the contracted condition. That the pulsations were occurring at a time when the cells were under the influence of the $CaCl_2$ is shown by the fact that the iridocytes throughout the entire scale were colorless. Attempts were also made to study the effects of various drugs upon erythrophore pulsations but the results were not worthy of serious consideration since no adequate means of controlling the experiments could be found. Comparison between two scales, even adjoining scales from the same fish, were quite useless as there was no constant uniformity in respect to the erythrophore behavior of the two. Scales were even cut into two halves, one half being immersed in solutions of the drug to be tested and the other half in NaCl as a control, but again no reliance could be placed on the data from a quantitative standpoint as control experiments showed that the erythrophores of the two halves of the same scale when placed in NaCl differed markedly in their behavior.

From these results and the results of previous investigation (von Frisch, 1912; Smith and Smith, 1934) it is apparent that in certain fishes the erythrophores are clearly under nervous control. In forms such as *Phoxinus* (Giersberg, 1930) it is equally clear that the erythrophores are independent of nervous control and are governed in their reactions by humoral factors. It still remains to be seen whether this bears any relation to the fact previously noted by us that in those forms where the erythrophores are under partial or complete humoral control their principal function seems to be their contribution to the assumption of the nuptial coloration shown during the spawning season. At other times they are relatively inactive. In forms such as *Holocentrus* and *Scorpæna* where a nervous control has been demonstrated the erythrophores are continually active at all seasons of the year and are in fact the dominating pigment cell among the chromatophores these animals possess.

In *Holocentrus* the erythrophores and xanthophores are alike in their behavior, both types of cells reacting about the same throughout all of the experiments attempted although possibly the xanthophores were somewhat more sluggish than the erythrophores in their responses to temperature changes. In *Scorpæna,* however, the xanthophores differ markedly from the erythrophores in that they show no pulsations in NaCl after treatment with $BaCl_2$ as do the erythrophores and are also much more sluggish in regard to their responses to other types of stimuli. Schnakenbeck (1925), after an examination of eighty different species of fish, came to the conclusion that there was no fundamental difference between xanthophores and erythrophores, in many cases both types of pigment being present in the same cell. On physiological grounds, however, such a generalization does not appear to be justified, although it may well be true of some fishes (i.e., *Holocentrus*). Any comparison between erythrophores and xanthophores other than in the same species seems useless until it is definitely established whether or not there are two distinct types of erythrophores, first the sluggish humorally controlled kind found in *Phoxinus* and second the highly sensitive nervously controlled type found in *Holocentrus.* Whether the difference between the two is actually clean cut, or whether the apparent distinction is only illusory as a result of the study of two extreme types in a graded series is a matter for further investigation.

Further complexity is again suggested when the melanophores and the erythrophores are compared. In *Scorpæna* the two types of pigment cells seem to behave essentially the same, differing only on morphological grounds. In *Holocentrus,* on the other hand, the melanophores vary pronouncedly from the erythrophores not only in appearance, for this they do also in *Scorpæna,* but also in responsiveness to certain types of stimuli. In *Holocentrus* the melanophores do not pulsate spontaneously in NaCl when transferred directly from sea water. In this species the erythrophores were by far the most active type of pigment cell present in the scale. The melanophores appeared to be sluggish in comparison, although the melanophores of *Holocentrus* were no more sluggish in their behavior than the melanophores of other forms. But the extreme sensitivity of the erythrophores of *Holocentrus* might well be anticipated after witnessing the rapidity with which this animal can adapt itself to a new shade of background. Apparently this form possesses a pigment cell which is fully as responsive as the chromatophores of lizards and in fact approaches the sensitivity of the entirely distinct pigmentary effector organ of the cephalopod.

SUMMARY

1. In the common Bermudian squirrel fish, *Holocentrus ascensionis* (Osbeck), sympathetic pigment-motor fibers can be demonstrated which are capable of altering the state of pigment distribution within the erythrophores.

2. In the denervated trunk erythrophores of *Holocentrus* an increase in temperature will cause a withdrawal of the pigment into the central body of the cell. Lowering the temperature has the opposite effect. In innervated trunk erythrophores the effect of temperature changes upon the state of pigment distribution of the erythrophore is the reverse of what is seen in denervated erythrophores.

3. Isolated scale erythrophores and xanthophores of *Holocentrus* show spontaneous pulsations when transferred from sea water to N/10 NaCl. Isolated scale melanophores will not pulsate in NaCl unless previously treated with N/10 $BaCl_2$.

BIBLIOGRAPHY

BALLOWITZ, E., 1913. Über die Erythrophoren in der Haut der Seebarbe, Mullus L., und über das Phänomen der momentanen Ballung und Ausbreitung ihres Pigmentes. *Arch. f. mikr. Anat.*, Abt. 1, **83**: 290.

VON FRISCH, K., 1911. Beiträge zur Physiologie der Pigmentzellen in der Fischhaut. *Arch. ges. Physiol.*, **138**: 319.

von Frisch, K., 1912. Ueber farbige Anpassung bei Fischen. *Zool. Jahrb.*, **32**: 171.

Giersberg, H., 1930. Der Farbwechsel der Fische. *Zeitschr. vergl. Physiol.*, **13**: 258.

Pouchet, G., 1876. Des changements de coloration sous l'influence des nerfs. *Jour. de l'Anat. Physiol.*, **12**: 1–90, 113–165.

Schnakenbeck, W., 1925. Vergleichende Untersuchungen über die Pigmentierung mariner Fische. *Zeitschr. f. mikr.-anat. Forsch.*, **4**: 203.

Smith, D. C., 1928. The Effect of Temperature on the Melanophores of Fishes. *Jour. Exper. Zool.*, **52**: 183.

Smith, D. C., 1930. Melanophore Pulsations in the Isolated Scales of Fundulus heteroclitus. *Proc. Nat. Acad. Sci.*, **16**: 381.

Smith, D. C., 1931. The Influence of Humoral Factors upon the Melanophores of Fishes, Especially Phoxinus. *Zeitschr. f. vergl. Physiol.*, **15**: 613.

Smith, D. C., 1933. Color changes in the isolated scale iridocytes of the squirrel fish, Holocentrus ascensionis. *Proc. Nat. Acad. Sci.*, **19**: 885.

Smith, D. C., and M. T. Smith, 1934. Observations on the Erythrophores of Scorpæna. *Biol. Bull.*, **67**: 45.

Spaeth, R. A., 1916. Evidence Proving the Melanophore to be a Disguised Type of Smooth Muscle Cell. *Jour. Exper. Zool.*, **20**: 193.

STUDIES OF LIVING NERVES[1]

IV. GROWTH, REGENERATION, AND MYELINATION OF PERIPHERAL NERVES IN SALAMANDERS

CARL CASKEY SPEIDEL

(From the Department of Anatomy, University of Virginia Medical School, University, Virginia)

In the earlier papers of this series of studies, the writer has presented observations of nerve activities as seen in living frog tadpoles (Speidel, 1932, 1933, and 1935). Case histories of individual nerve fibers over long periods of time have been obtained. These have revealed fundamental movements and changes that occur during the growth of nerves, during the formation of the myelin sheath, and during nerve irritation, degeneration, and repair.

Up to the present time frog tadpoles are the only forms in which nerve changes have been seen by this method of direct observation *in vivo*. It seemed desirable that a similar study be made on some representative of another group of vertebrate animals. Preliminary observations on a number of species of urodele amphibians and fishes indicated that it was possible to study living nerves in some of these. The present investigation deals with the behavior of the peripheral nerves in the tail of young salamanders during growth, regeneration, and myelination.

MATERIAL AND METHOD

The best observations were obtained on young salamanders (*Triturus viridescens*) during the fall of the year. These were approximately seven or eight months old. An average specimen was 30 mm. in length. Animals of other ages, however, were also watched. These included young larvae recently hatched from the eggs, and a few animals more than a year old.

The animals were examined microscopically under light chloretone anesthesia in an upright paraffin chamber. Between observations they were kept isolated in jars containing pond water and vegetation conducive to growth. The finer details of structure were observed with the aid of a 3-mm. oil-immersion objective lens.

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[1] This work has been aided by grants from the National Research Council, from the Committee on Scientific Research of the American Medical Association, and from the Research Committee of the Virginia Academy of Science.

Distribution of Nerves in the Tail of the Salamander

The central portion of the tail is occupied by the spinal cord, axial skeleton (notochord), and the muscle masses. This region is not suitable for observation in the living animal. The spinal cord extends almost to the tip of the tail. Emerging from between the muscle masses may be seen the cutaneous nerves that supply the tail fin expansion. Many of these are of the mixed type consisting of both myelinated and unmyelinated fibers. Others are entirely of the unmyelinated type. Occasionally, a single myelinated fiber may be seen which is isolated from other fibers for a part of its extent. Myelin-emergent sprouts are often intimately intermingled with non-myelin-emergent fibers. Sometimes, however, they are separate and can be traced to their terminations just beneath the basement membrane of the epithelium.

The lateral line nerve and its branches constitute a conspicuous part of the peripheral nervous system in the tail. Rami of this nerve, which is itself a division of the vagus nerve, are particularly prominent in the dorsal fin region. These fibers innervate the many lateral line organs of special sense which are located in the tail.

Essentially, the peripheral nervous system of the tail of the young salamander is like that of the frog tadpole. A few minor differences, however, may be noted. In the salamander the sheath cells of Schwann are larger, the myelin segments longer, and the myelinated fiber is of smaller caliber; all of the regenerative activities proceed at a slower rate; the sheath cells migrate and proliferate less rapidly; the ameboid growth cones travel at a slower pace; and the process of myelin ensheathment requires more time.

Growth of Individual Nerve Sprouts and the Formation of the Early Unmyelinated Nerves

Pioneer Growth Cones

Experimentally many actively growing nerve sprouts may be induced by subjecting the animal to partial tail amputation followed by several days of regeneration. Careful examination of the newly regenerating zone reveals the expanded growing tips of single nerve fibers. These terminal enlargements are called growth cones. Each pioneer growth cone probes its way slowly through the mesenchymal tissues spinning a nerve fiber behind it. The growth cones migrate by ameboid movement. Delicate pseudopods are frequently extended and retracted. At times tiny refractive granules are visible.

Progress, though rapid at times, is somewhat sporadic. Slight barriers, such as connective tissue cell processes, may cause temporary halting and varicosity formation. A change in the direction of growth of the nerve fiber may follow. Growth cones at rest usually draw in their pseudopods and become smoothly rounded. Retracting nerve tips are characterized by the appearance of knob-like excrescences or vesicles.

Typical behavior of a pioneer growth cone over a period of one hour is shown in the illustration (Fig. 1). Extension, temporary varicosity formation, constriction ring formation, deflection by a connective tissue cell process, and variations in pseudopods and in granular content are all visible in this example. A distance of about 20 micra was traversed during the hour.

The group of pioneer growth cones shown in the illustration (Fig. 2) exemplifies another principle which is of some theoretical interest. In spite of the close proximity of the growth cones to one another with presumably very similar local environment, their behavior varies. While some exhibit definite retraction, others actively advance or show no marked change in position. This suggests that there must be a distant central influence over nerve sprout advance; that, though local conditions may be important, they are not the sole determiners of the behavior of the sprouts.

In one of the other case histories (Fig. 4) the behavior of several growth cones over a period of three days is shown. This case suggests

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PLATE I

The following figures are based upon free-hand sketches, and they are, therefore, not entirely accurate. In all figures *proximal* is toward the left (or below), and *distal* toward the right (or above).

FIG. 1. The progress of a growth cone of a nerve fiber over a period of one hour. Salamander, No. 882, regenerating zone eight days after partial tail amputation.

11:30 A.M., the growth cone, *S*, is slightly obstructed by the fibroblast process, *P*. 11:37 to 11:40, the growth cone passes the obstruction, a constriction ring being present at the point. 11:51 to 12:08, a second connective tissue cell process is passed which is located somewhat obliquely to the course of growth of the nerve fiber. 12:25 to 12:30, a third process of a connective tissue cell is approached.

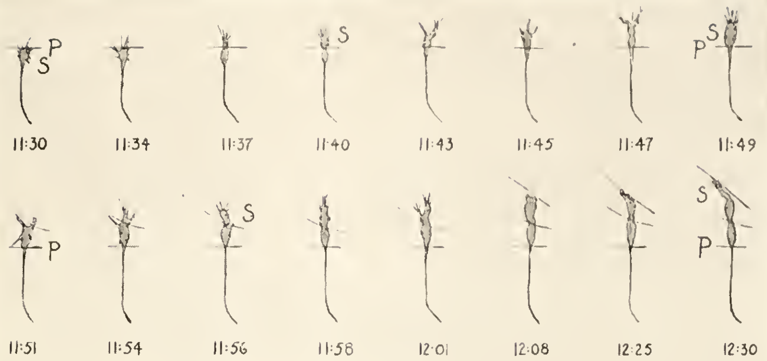
FIG. 2. Movements of extension and retraction shown by several free growth cones within a small area. Salamander, No. 881, eight days after partial tail amputation.

Five growth cones are visible at *P*, *Q*, *R*, *S*, and *T*. During thirty-five minutes of observation growth cones *P*, *R*, and *T* advanced somewhat, while *Q* and *S* both retracted.

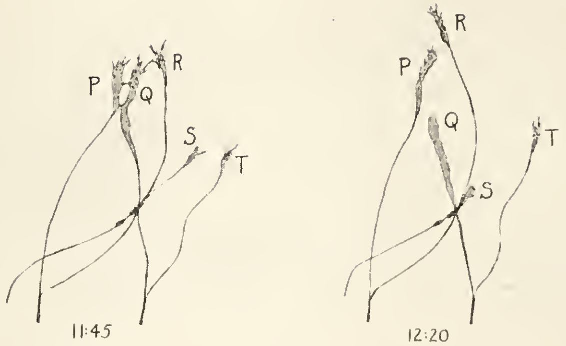
FIG. 3. The extension of the first three growth cones of a young nerve over a period of one hour. Salamander, No. 882, regenerating zone, eight days after partial tail amputation.

At 11:35 two growing tips were visible at *P* and *Q* advancing slowly through the tissues toward the edge of the tail fin. A third growth cone at *R* was diverging slightly from the line of the nerve.

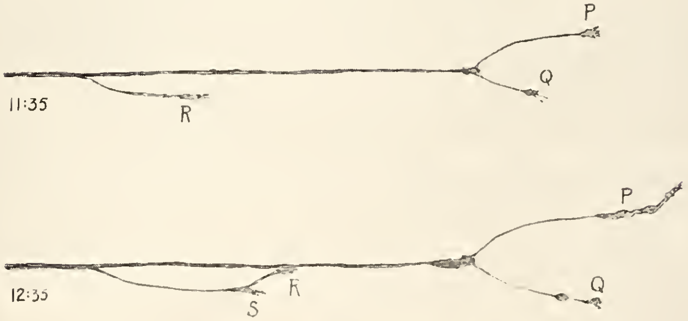
12:35, one hour later *P*, *Q*, and *R* all showed some progress and at *S* a new growth cone appeared.


S
P
11:30
11:34
11:37
S
11:40
11:43
11:45
11:47
P
S
11:49
P
11:51
11:54
S
11:56
11:58
12:01
12:08
12:25
S
P
12:30

1


P
Q
R
S
T
11:45
R
P
Q
S
T
12:20

2


P
Q
11:35
R
P
Q
12:35
S
R

3

Plate I

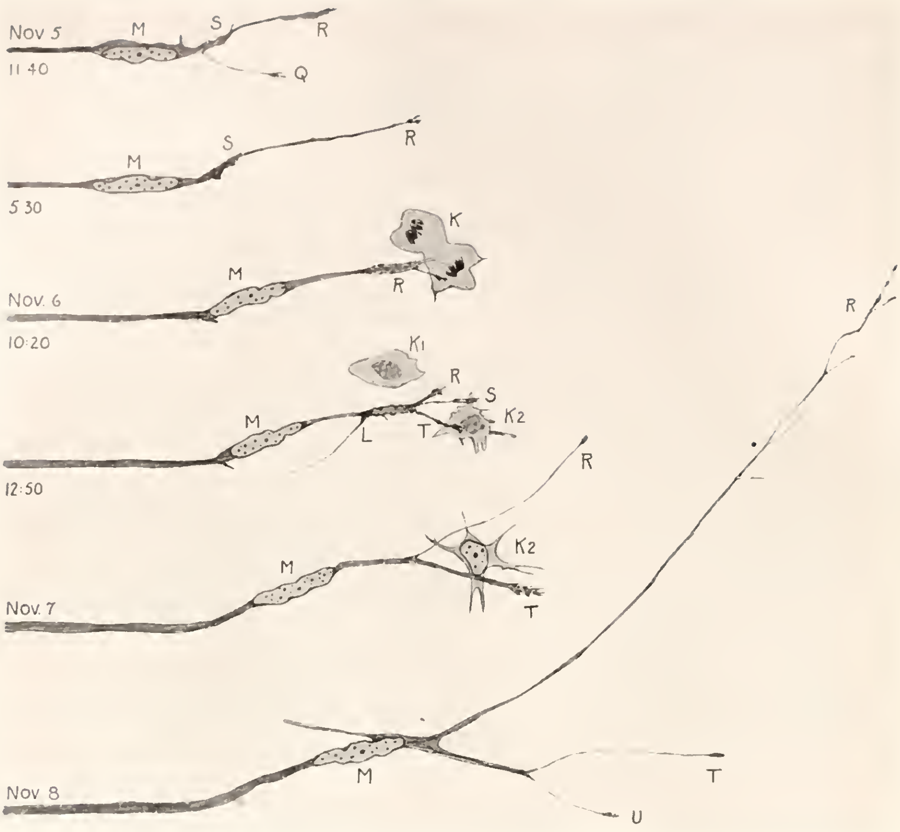


Plate II

Fig. 4. Movements of growth cones and a sheath cell of Schwann over a period of three days; the formation of branches correlated with a nearby fibroblast mitosis. Salamander, No. 881, regenerating tail fin observed from eight to eleven days after partial tail amputation (cut October 28).

November 5, 11:40 A.M., three growth cones, *Q*, *R*, and *S* are visible. The primitive sheath cell, *M*, has reached the position shown and exhibits slow movements with little change in position.

November 5, 5:30 P.M., the small branch which ended in *Q* has disappeared. *R* has advanced.

November 6, 10:20 A.M., sheath cell *M* has advanced. *R* is swollen and in close relation to the dividing fibroblast, *K*. Growth cone, *S*, is indistinguishable but is probably present in the swollen tip at *R*.

November 6, 12:50 P.M., three separate growth cones, *R*, *S*, and *T* advance as the cell division becomes completed with the formation of the daughter cells, *K*1 and *K*2. Another growth cone touches the nerve at *L*, causing some agitation of the neuroplasm at this point.

November 7, *R* and *T* have advanced, but *S* has disappeared. The connection at *L* has also been lost. The position of *K*2, one of the daughter cells of the fibroblast division of the preceding day, is shown.

November 8, *M* advances somewhat. And at its distal end a new connection with another nerve is present. Growth cones, *R* and *T*, show further extension and a new branch develops which ends in the growth cone at *U*. The nerve increases in diameter.

that mitosis of an adjacent fibroblast has a stimulating effect on nerve sprouts. It also indicates that the neuroplasm of a young nerve is responsive to the touch of an active growth cone. Similar observations have been recorded in the case of frog tadpoles (Speidel, 1933 and 1935).

Later Growth Cones

Following the first nerve fibers come the second, third, and later growth cones, each spinning a fiber of its own. These usually follow more or less closely the pathway laid down by the pioneer growth cones. In this manner the first small unmyelinated nerves are formed.

In the example given (Fig. 3) a small regenerating nerve is shown which toward the left consists of three fibers. The terminal growth cones of these fibers are visible at *P*, *Q*, and *R*. Over a period of an hour each of these advances somewhat. The origin of a new sprout from one is visible as a new growth cone forms at *S*.

The Primitive Sheath Cells of Schwann

The early small nerves which at first consist of only a few naked fibers become quickly provided with satellite cells, the primitive sheath cells of Schwann (neurilemma cells).[2] These cells migrate distally along the nerves by ameboid movement. The example given (Fig. 4) shows the rather slow progress of the most peripheral sheath cell on a small nerve over a period of three days.

In regenerating zones the Schwann cells often divide by mitosis (Fig. 5). While at rest these cells are much elongated and flattened along the nerve. In preparation for division, however, they slowly swell and round up. The fast motion ciné-photomicrographs vividly reveal the violent agitation that takes place during the actual division into two cells. Distinct local swelling of the nerve usually accompanies the process. The daughter cells then move apart and slowly flatten out to assume their typical elongated resting position along the nerve.

A sheath of Schwann (neurilemma), syncytial in nature, is elaborated. This sheath is tubular and at first usually encloses *several* nerve fibers. Septa may develop later to effect separate ensheathment for single fibers.

THE PROCESS OF MYELIN SHEATH FORMATION

As nerve fibers become more mature many acquire a myelin sheath. This sheath is laid down in segments. The first segments appear near

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[2] Embryologically, the sheath cells of Schwann are derived from ectoderm. They originate from the neural crest of the developing spinal cord, migrate outward along the dorsal roots, and then follow the spinal nerves.

the nerve roots. The progress of myelination is from proximal to distal. Each new segment is added to the end of the myelin line. As in frog tadpoles, a nerve fiber emerging from a myelin segment (myelin-emergent fiber) exhibits a greater bias toward myelin formation than one which does not emerge from a myelin sheath (non-myelin-emergent).

Myelin-emergent and Non-myelin-emergent Fibers

Careful examination of nerves near the transition between a proximal myelination zone and a distal non-myelination zone reveals that myelin-emergent sprouts may sometimes be distinguished from the non-myelin-emergent fibers which make up the bulk of an ordinary unmyelinated nerve. In the example given (Fig. 6) extremely delicate myelin-emergent sprouts emerging from the last myelin segment are visible. They are quite distinct from the fibers of the accompanying unmyelinated nerve which is provided with a sheath cell of Schwann and a neurilemma. The myelin-emergent sprouts are without a sheath of any sort and are, therefore, quite naked. In this particular instance no further myelination took place although the region was observed for 60 days.

As a rule, however, a myelin-emergent fiber which is ripe for ensheathment is intimately associated with accompanying non-myelin-emergent fibers, often being within the same neurilemma at first. For this reason, it is ordinarily not distinguishable. In all of the following examples it is noteworthy that each new myelin unit is formed as a result of the coöperative activity of a sheath cell and a nerve fiber, particularly a myelin-emergent fiber.

Addition of Two Terminal Myelin Segments

In the example (Fig. 9) a small mixed nerve consists of a single fiber in process of myelination and an unmyelinated nerve which is made up of several nerve fibers. To the right of the arrow in the direction of the young sheath cell *S*, the unmyelinated nerve includes both a myelin-emergent fiber and several non-myelin-emergent fibers. Proliferation of the primitive sheath cells is followed closely by the formation of two new myelin sheath segments. Marked swelling of the myelin-emergent fiber accompanies the process.

Comparison of the young myelin segments in the last two sketches brings out the slow but steady growth in both diameter and length of the myelin units.

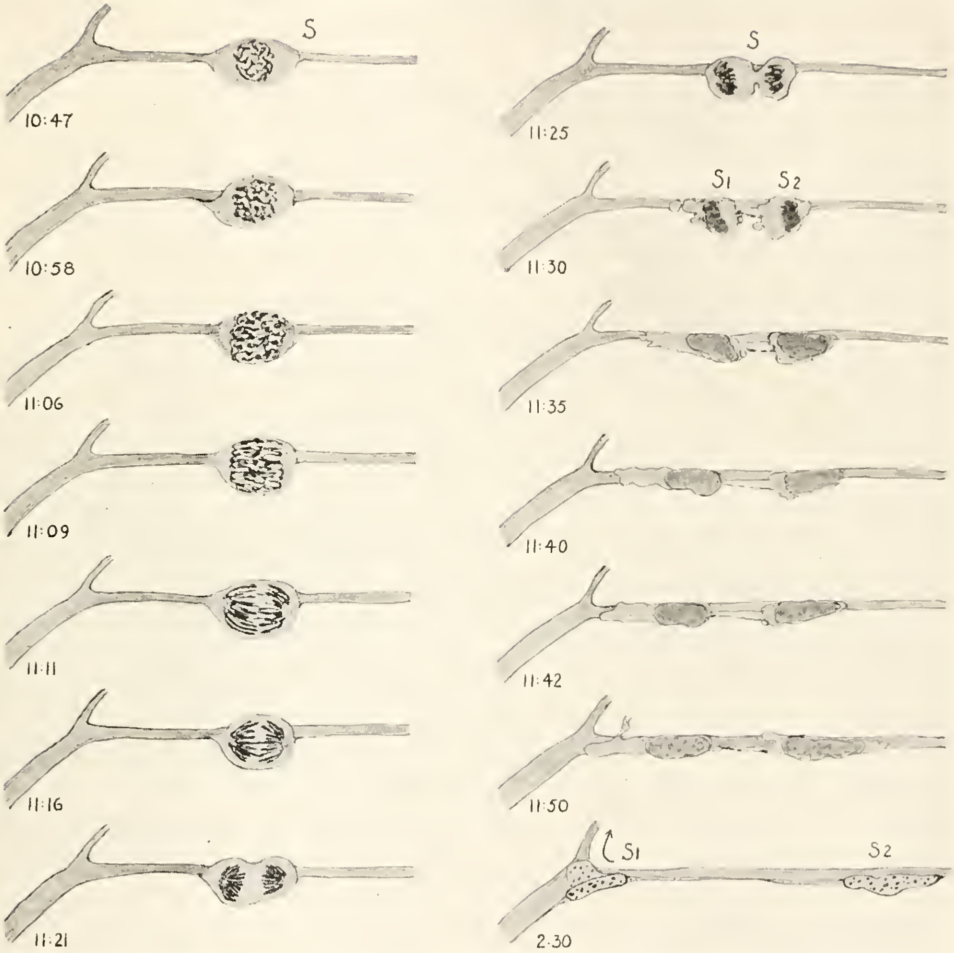

S
10:47
10:58
11:06
11:09
11:11
11:16
11:21
S
11:25
S1
S2
11:30
11:35
11:40
11:42
11:50
S1
S2
2:30

Plate III

Fig. 5. Sheath cell division on a small peripheral unmyelinated nerve. Salamander, No. 880, regenerating tail fin ten days after partial tail amputation.

During the hour from 10:47 A.M. to 11:50 A.M. the changes in a dividing sheath cell are shown. At 11:51 the animal was replaced in pond water. At 2:30 P.M. the two daughter cells were separated as shown. During the next day the cell, *S*1, migrated out of the upper fork of the nerve in the direction indicated by the arrow.

Intercalation of a Myelin Segment Between Two Others

Sometimes myelination along a fiber takes place somewhat irregularly, in such a way that temporary bare lengths are left in some places between two myelin segments. Such bare lengths of fiber, if they are not very extensive, are usually covered by growth of the adjacent myelin units (cf. Fig. 10, growth of segment *T* toward *U*).

If the unmyelinated portion is too extensive, however, the process of ensheathment is accomplished by the intercalation of an entire myelin unit with its own sheath cell. In the example given (Fig. 10) between the myelin units at *R* and *T* is a region without myelin. Since a primitive sheath cell *S* is located in this region, conditions are favorable for myelination. Within two days a young segment is intercalated which then extends during the next two days to reduce the unmyelinated gaps at each end.

Plate IV

Fig. 6. A small mixed nerve showing the intimate relation of a myelin-emergent fiber with the accompanying non-myelin-emergent fibers. Normal salamander, No. 878.

From the terminal myelin segment, *T*, emerges a fiber which divides into naked sprouts at *S*. One of these is visible as it extends in close association with the accompanying unmyelinated nerve toward the sheath cell, *R*. Several of the sprouts terminate immediately beneath the basement membrane of the skin epithelium.

Fig. 7. Rapid swelling and ovoid segmentation of a segment of the myelin sheath following a hot water burn. Salamander, No. 997.

12:15, a normal myelin sheath segment. At 12:18 the tip of the tail, close to the segment illustrated, was immersed in hot water for a few seconds.

12:20, both the myelin segment and the sheath cell nucleus have become greatly swollen.

12:22, the sheath breaks up into myelin ovoids which remain connected for a time.

12:30, a later stage.

Fig. 8. Appropriation of a portion of one myelin segment by the next one following end-to-end anastomosis and establishment of a new node of Ranvier. Regenerating zone of salamander, No. 867, following tail section on October 12. The segments of this figure are the same ones illustrated in Fig. 12, segments *N*, *O*, and *P*.

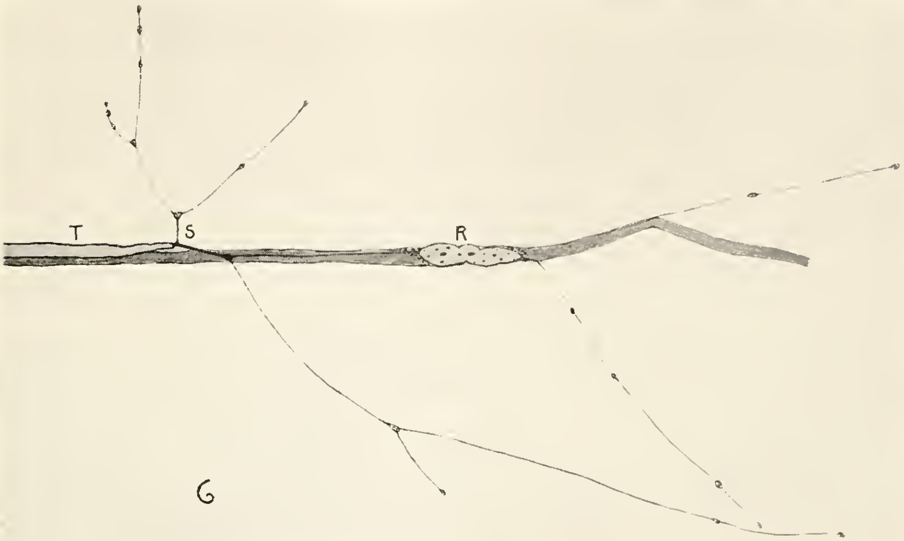
November 24, a bare length of fiber without a myelin sheath lies between the myelin segments associated with *O* and *P*.

December 6, sheath cell, *O*, moves toward the bare length. Both segments grow in length.

December 19, the myelin segments become joined. Thus, two sheath cells are located for a number of days on a single fused segment.

January 6, sheath cell, *P*, migrates toward the right beyond the field illustrated and a new node of Ranvier develops between *O* and *P*. The myelin sheath between the two arrows *originated* under the influence, not of sheath cell *O*, but of sheath cell, *P*.

During the same period a new terminal segment forms under the influence of *Q*1 along the upper nerve fork, following a division of the young sheath cell *Q*. Movements of sheath cells, *N*, *P*, and *Q*2 are also evident over the 4-day period illustrated.



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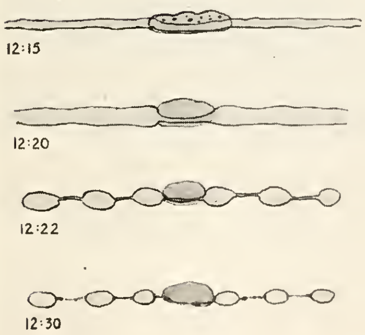

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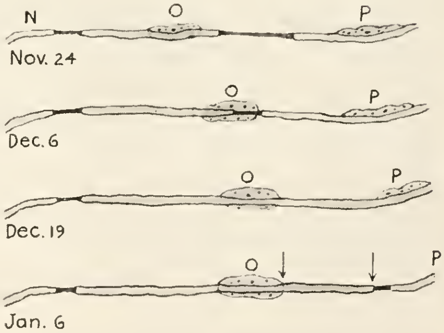

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PLATE IV

Appropriation of a Part of a Myelin Segment by the One Next to It, Following an End-to-end Anastomosis of the Two Segments

Occasionally, myelin is elaborated at the primitive node of Ranvier between two young segments. This results in fusion of the two segments with complete obliteration of the node (Fig. 8). The elongated segment is left for a time with two sheath cells. In the example cited, one of the sheath cells moved distally for a short distance and a new

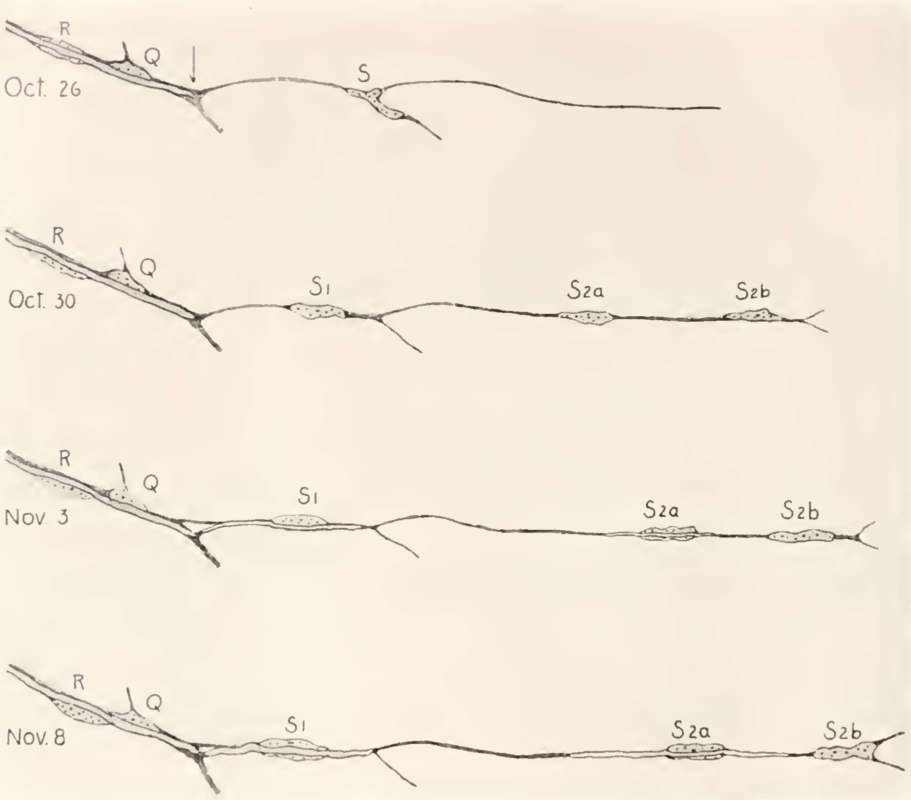

Plate V

Fig. 9. Addition of two new myelin sheath segments at the end of a myelinating fiber in a normal young salamander, No. 869.

October 26, the myelin sheath of a single myelinated fiber ends at the point indicated by the arrow. The sheath cell, *R*, is associated with the final myelin segment. The sheath cell, *Q*, is on the accompanying unmyelinated nerve. The most peripheral sheath cell on the nerve is shown at *S*.

October 30, *S* divides once, giving rise to *S*1 and *S2* and the latter daughter cell divides giving rise to *S2a* and *S2b*.

November 3, two new myelin segments are formed, one associated with *S*1 and the other with *S2a*.

November 8, the new segments grow somewhat both in length and in diameter and in thickness of the myelin sheath.

This region was kept under observation for more than two months longer. During this period the animal was subjected to partial starvation and no further essential growth changes took place. Finally, on January 18, the myelin segment associated with *S2a* suffered degeneration, possibly as an indirect result of malnutrition.

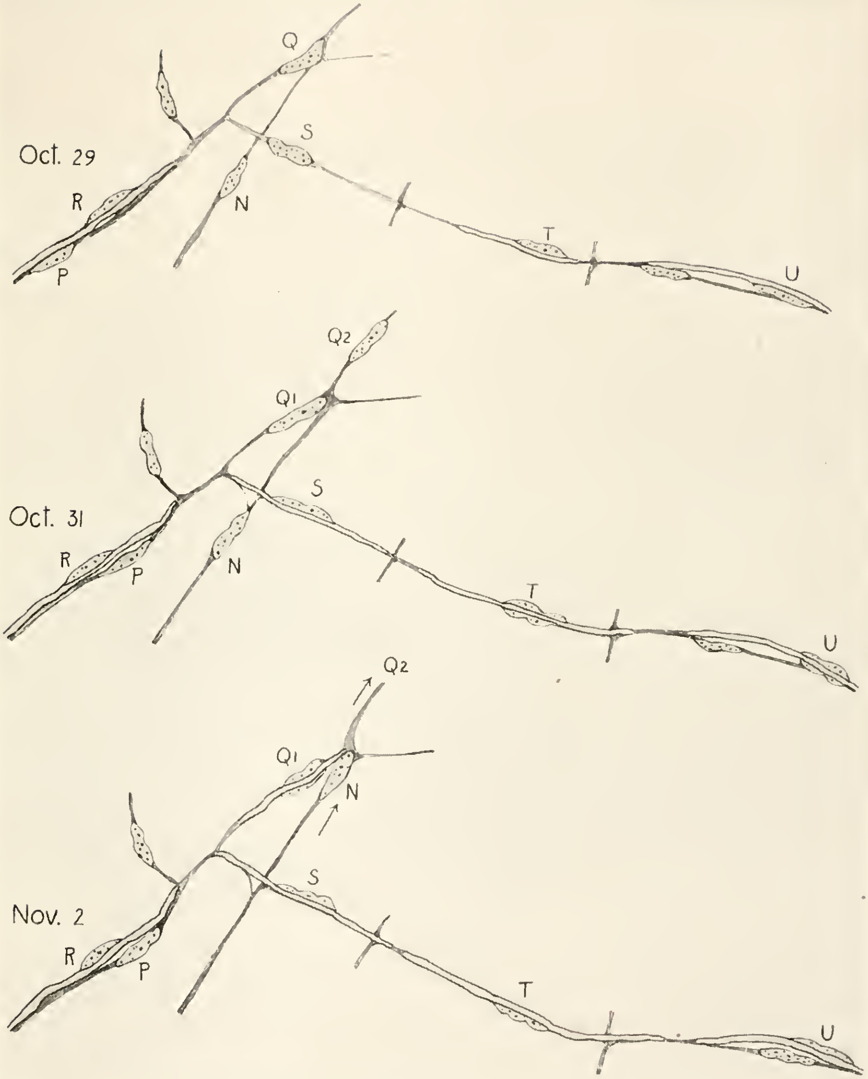

Plate VI

Fig. 10. Intercalation and addition of new myelin sheath segments in the vicinity of a nerve fork. Normal salamander, No. 876.

October 29, three myelin segments are visible associated with the sheath cells, *R*, *T*, and *U*, respectively. Sheath cells, *P*, *Q*, *S*, and *N* are associated with unmyelinated nerve fibers.

October 31, a new myelin segment appears in relation with *S*. Longitudinal extension of the myelin sheath segments associated with *R* and *T* is also noticeable. *Q* divides, giving rise to *Q*1 and *Q*2.

November 2, a new myelin segment is formed in relation with *Q*1. The segment associated with *S* grows both in length and in diameter.

node of Ranvier then appeared at a locus previously covered with myelin. In this manner a segment was left provided with myelin which had originated under the influence of two sheath cells.

Rapid Myelinogenesis During Regeneration

The preceding examples of myelin segment formation are from animals undergoing slow normal growth and development. A very rapid process of myelination may be induced experimentally by partial amputation of the tail, followed by regeneration. From the cut ends of the nerves at the site of amputation sprouts grow out into the newly regenerating tail tip, the sheath cells of Schwann follow along them, and typical unmyelinated nerves are formed. Lines of myelin segments appear later along some of the fibers.

In the example given (Fig. 12) a region is shown in the newly regenerating tail fin 11 days after the amputation operation. During the next 7 days the nerves increased in size and the sheath cells proliferated and moved distally as indicated by the arrows. That some of the fibers were ripe for myelination is indicated by their swollen appearance and by the alignment of sheath cells along the edge. On the following day many new definitive myelin segments were visible. During the next three weeks further progress of myelination took place. This consisted not only in the formation of new segments, but also in the

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Plate VII

Fig. 11. Pressure irritation and degeneration of terminal myelin segments, followed by temporary restoration of one and loss or transfer of sheath cell. Normal salamander, No. 866.

October 23, at the end of a single nerve fiber four normal myelin segments are visible associated with their sheath cells, *Q*, *R*, *S*, and *T*, respectively. On October 26, as a result of slightly too much pressure exerted by the cover slip during observation, these segments became greatly irritated and degeneration ensued.

October 30, sheath cell, *T*, disappears entirely. Sheath cell, *S*, has transferred to a near-by nerve. Sheath cells, *Q* and *R*, remain in position. Small myelin ovoids and granules are in evidence along the degenerating fiber. New sprouts appear at *N* and at *J*.

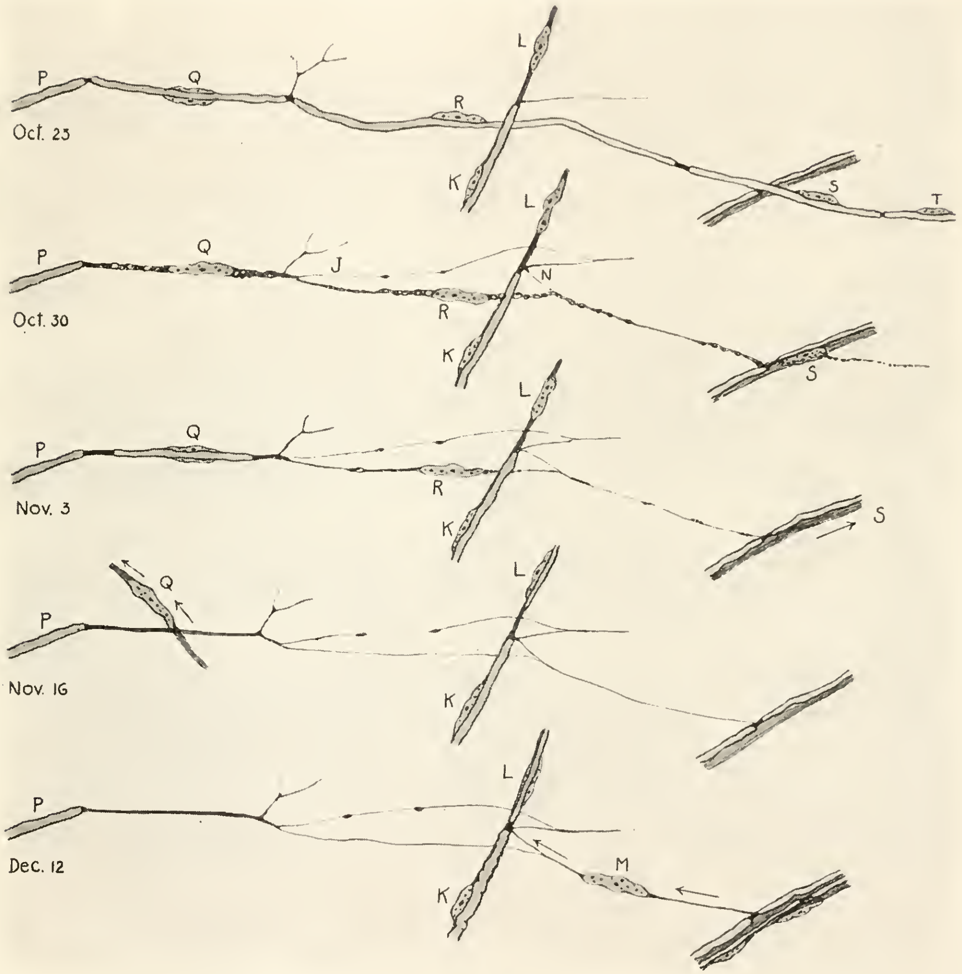
November 3, in association with sheath cell, *Q*, a new myelin segment forms. It grows in length during the next few days. Sheath cell, *S*, migrates farther distally along the nerve, as indicated by the arrow. On November 6 the sheath cell, *R*, becomes somewhat vacuolated and then undergoes degeneration. On November 12 the newly regenerated myelin segment associated with *Q* degenerated.

November 16, sheath cell, *Q*, transfers to a near-by nerve and slowly migrates in the direction of the arrow. A new myelin segment appears in association with *L*.

December 12, a sheath cell, *M*, transfers from the nerve at the right and moves as indicated by the arrows, finally arriving at the node of Ranvier between segments, *K* and *L*. A second line of myelin segments appears on one of the fibers of the mixed nerve at the right of the figure. The wrinkling of the myelin sheath of the segment associated with *K* is indicative of an irritated state. On January 3 this segment degenerated.

growth in length and diameter of each unit, and increase in the thickness of the myelin.

In the large nerves the intimate intermingling of the fibers in process of myelin ensheathment with those that remained unmyelinated prevented clear-cut histories for all segments. In some cases, however, the complete history of formation and growth of single segments is obvious (as with *M*, *R*1, *T*, and *U*).


Oct. 23
P
Q
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K
S
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P
Q
J
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N
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Nov. 3
P
Q
R
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P
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K
Dec. 12
P
L
K
M

Plate VII

This example of regeneration, together with others of similar nature,

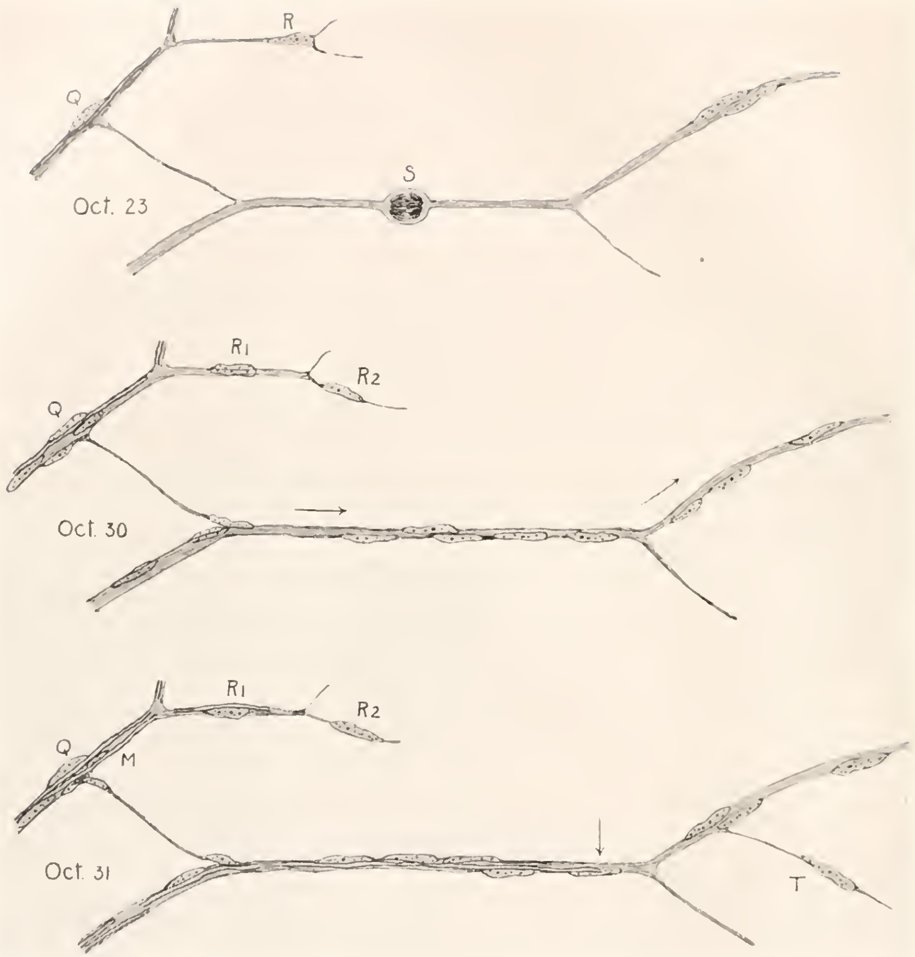

Plate VIII (a)

Fig. 12. Myelinogenesis during rapid regeneration. Salamander, No. 867, regenerating zone, 11 to 40 days after partial tail amputation. The tail was sectioned on October 12.

October 23, a sheath cell, *S*, on a newly regenerating unmyelinated nerve is in mitosis. Two other sheath cells are visible on this nerve distally. The mixed nerve at the upper left of the figure includes an unmyelinated portion and a single myelin segment associated with the sheath cell at *Q*.

October 30, the sheath cells multiply and exhibit migration distally, as indicated by the arrows. Sheath cell, *R*, divides into *R*1 and *R*2. Both nerves have a somewhat swollen appearance and there is a distinct tendency for the sheath cells to align themselves along the nerve edges.

October 31, two lines of thin myelin segments become differentiated in the lower nerve extending distally to the point indicated by the arrow. In the upper nerve a second fiber becomes provided with a segment appearing at *M* and another at *R*1. A new sprout and sheath cell appear at *T*.

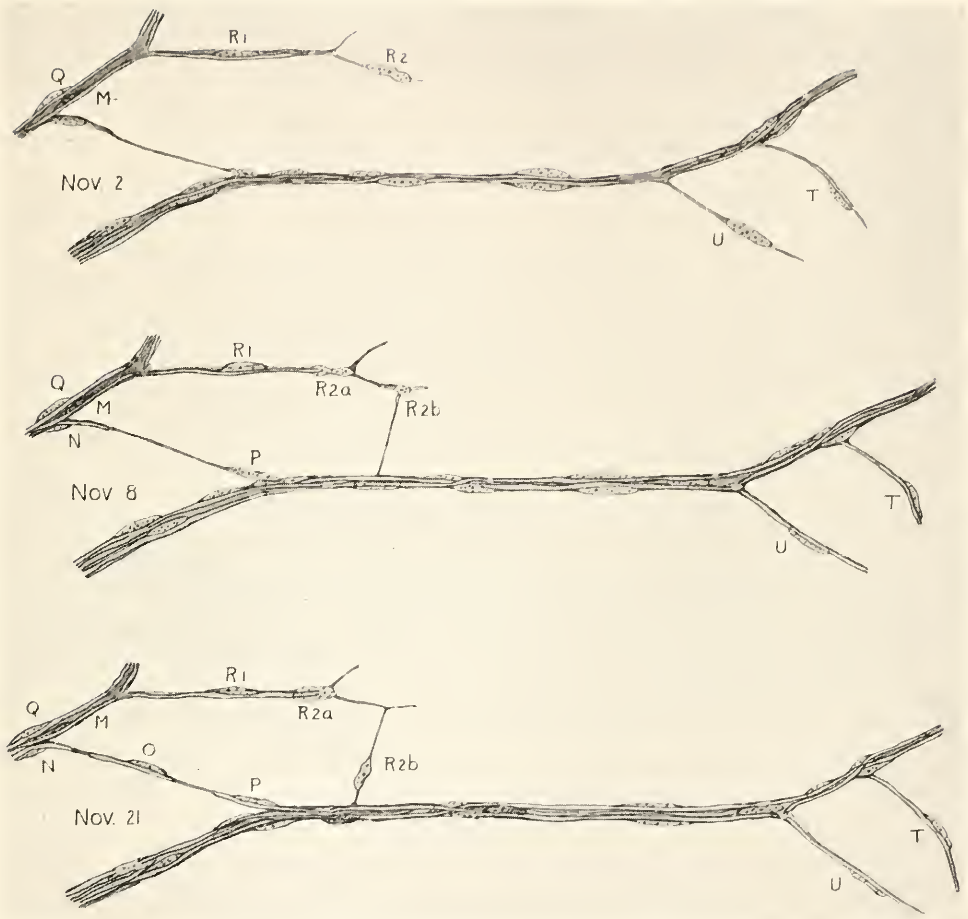

Plate VIII (b)

November 2, myelin segment formation proceeds distally. A new segment is formed at *T*.

November 8, the myelinated fibers become more conspicuous and many of the separate segments exhibit growth in length and thickness. New myelin segments are visible at *U* and at *N*.

November 21, the lower nerve now includes three myelinated fibers as well as an unmyelinated portion. Elongation of segments takes place which is correlated with the growth of surrounding tissues as regeneration of the tail proceeds. New myelin segments are visible at *O* and *P*.

This region was kept under observation until January 18, during which time some further minor changes took place. Segments, *O* and *P*, presented a history of special interest, which is shown in Plate IV, Fig. 8.

makes it clear that the great time for new myelin segment formation in the proximal regenerating zone is from about 18 to 25 days after the injury. In more distal zones of regeneration this may be somewhat later.

This case also brings out how whole nerves lengthen or stretch to keep pace with the growth of the terrain. Thus, the distance between the two forks of the lower nerve becomes distinctly greater during the 29 days represented by the sketches (Fig. 12).

Myelination of a Cranial Nerve

Branches of the lateral line nerve (a vagus nerve division) were watched to see whether there might be any difference in the mechanism of myelin-sheath formation along a cranial nerve fiber. Case histories of a few myelin segments were obtained which indicated that the process of myelination on this cranial nerve is entirely like that observed on the spinal nerves.

Nerve Irritation and Adjustment

During growth and regeneration the nerves are subjected to various stresses and strains. Nerve irritation is frequently the result. A condition of this sort is easily recognizable because of the reaction exhibited by the myelinated fibers.

An irritated internodal segment may exhibit swelling, vacuole formation between the axis cylinder and the myelin sheath, widening of the node of Ranvier as the myelin sheath slips or disappears in this region, and wrinkling of the myelin sheath. Myelin spherules may become detached from the sheath. If the irritation is strong the segment will degenerate, the sheath segmenting into myelin ovoids and granules. If the irritation is not too strong the segment may readily recover within a day or two, suffering little or no loss of its myelin sheath.

A beautifully graded series of irritated segments may be observed immediately after the tip of the tail is momentarily immersed in hot water. A nerve fiber which innervates the scalded zone shows acute irritation. On such a fiber the myelin segments near the edge of the burned region quickly swell and undergo rapid metamorphosis leading to the formation of myelin ovoids (Fig. 7).

Farther proximally the myelin segments are less strongly irritated. They exhibit a distinct swelling like that shown by the segment in the illustration (Fig. 7, at 12:20). They do not break up, however, and during the next few days, as recovery takes place, the swelling subsides.

Similarly, following partial tail amputation the myelin segments bordering the cut edge undergo traumatic irritation and degeneration.

Those farther proximally may remain quite intact and merely exhibit swelling. A case of traumatic degeneration following an injury to the overlying skin is given (Fig. 11). This case also illustrates replacement of one of the degenerated myelin segments, followed later by a second degeneration.

Whenever tissue shrinkage occurs, the nerve fibers in the zone of shrinkage become somewhat wrinkled. Sometimes a slight degree of telescoping of one segment on the next is evident. Swelling, vacuole formation, and detachment of small myelin spherules may accompany this condition of irritation. Recovery may, or may not, take place without loss of the myelin sheath, depending upon the strength of the irritation. Older more massive segments of the myelin sheath offer stronger resistance to the degenerative process than do younger slighter segments.

Typical phenomena of nerve irritation may also be observed after subjection of the animal to alcohol intoxication, to a strong anesthetic (chloretone), to pressure, to partial desiccation, to starvation or malnutrition, and to near-freezing temperature. Since many varieties and many gradations of nerve irritation of both acute and chronic types have already been described in some detail in the case of frog tadpoles (Speidel, 1935), it is unnecessary that further consideration be given to this subject in the salamander. Fundamentally, the reactions in both species are quite similar.

Degeneration and Regeneration Along the Proximal and Distal Stumps of Sectioned Nerves

After nerve section the processes of degeneration and regeneration in the salamander proceed as in the frog tadpole. Typical trophic (Wallerian) degeneration takes place along the distal stump. In the case of sectioned myelinated fibers the peripheral portion always exhibits total degeneration. A certain amount of traumatic degeneration is visible on the proximal stump close to the site of the wound. Repair is effected by growth of sprouts from the cut nerve fibers, by migration and multiplication of the sheath cells of Schwann, and finally by formation of new segments of myelin sheath. Ordinarily, the regenerating fibers follow the distal stump more or less closely.

The regeneration of sectioned unmyelinated nerves is more difficult to follow exactly. These nerves form anastomoses freely with one another. The distal stump of a cut unmyelinated nerve usually exhibits partial but not total degeneration. The explanation of this result, together with illustrative case histories, has already been advanced in connection with similar results in frog tadpoles. The distal stump of such a nerve includes at least one *reverse* fiber which has not been severed from its cell body by the cut, but is still connected with it by way of a peripheral anastomosis with a normal unsectioned nerve. As far as such a reverse fiber is concerned the distal stump is in reality a proximal stump. Furthermore, after the operation other fibers from adjacent nerves may join the degenerating distal stump and grow along it in either direction. Junction of distal and proximal stumps may take place with growth cones progressing in either direction across the wound zone. Collateral sprouts are usually stimulated to grow out from the near-by uninjured nerves.

Mixed nerves, likewise, since they include an unmyelinated portion, may exhibit incomplete degeneration as far as the unmyelinated fibers are concerned.

Polariscopic Observations

Under the micropolariscope with crossed Nicol prisms the myelin sheath is brilliantly illuminated. It is negatively anisotropic. The neurilemma, axis cylinder, the unmyelinated fibers, and the terminal growth cones are all isotropic. Occasionally, though not often, one or more anisotropic granules may be present within a sheath cell.

In the development of a myelin segment the pre-myelin substance is isotropic. Very young myelin segments exhibit thin lines of weak anisotropy. Older and thicker myelin segments exhibit strong anisotropy. Thus, the development of the anisotropic condition closely parallels the growth of the sheath.

During myelin sheath degeneration, the anisotropic condition is exhibited by the myelin ovoids and granules. Gradually the anisotropy becomes less pronounced as degeneration proceeds. In the case of individual globules or granules, however, it may persist for several days or more.

Ciné-Photomicrographs of Nerve Fibers in the Salamander

Successful motion-picture records have been obtained of salamander nerve fibers showing the essential changes during growth and myelination. The magnification is sufficient to reveal some of the finest histological details. These ciné-photomicrographs are mostly of the fast-motion (time-lapse) type.

Movements of the growing nerve tips are vividly revealed by pictures taken at the rate of one every two seconds. When these are projected on the screen at the rate of sixteen per second all movements become magnified thirty-two times. Movements of the sheath cells are quite slow and are best brought out on the screen by photographs taken at a slow rate, e.g., one every eight seconds. In projection these movements become magnified one hundred and twenty-eight times. For records of the daily progress of myelin sheath formation on a fiber, the pictures were usually taken at the normal rate of sixteen per second.

Among the subjects photographed are the following:

1. Rapidly advancing pioneer growth cones in newly regenerating regions (several examples).
2. Varicosity formation following the blocking of an active growth cone by connective tissue cell processes.
3. Movements of sheath cells of Schwann along newly regenerating nerves.
4. Mitoses of sheath cells of Schwann.
5. Addition of new terminal segments of myelin sheath along a regenerating nerve fiber.
6. Formation of many new myelin segments and the process of transformation of a composite unmyelinated nerve into a mixed nerve including several myelinated fibers. (Photographed at intervals from 16 to 71 days after partial amputation of the tail.)
7. Examples of strongly irritated myelin segments.

SUMMARY

In living salamanders (*Triturus viridescens*) individual nerve fibers have been directly observed for prolonged periods (several months) during the processes of growth and myelination, degeneration and regeneration, irritation and recovery. The behavior of nerves in the young salamander is fundamentally like that in the frog tadpole.

In newly regenerating zones a few days old the earliest nerve sprouts are visible slowly probing their way through the mesenchymal spaces toward the skin. At the tip of each fiber is an enlargement, or growth cone, which advances by ameboid movement. In action the growth cone is provided with delicate pointed pseudopods; while at rest it is smoothly rounded; during retraction it is characterized by knob-like excrescences.

Growth of the sprouts is not necessarily continuous, but is often sporadic in nature. That local conditions are not wholly responsible for growth cone progress is suggested by the differences in behavior exhibited by closely grouped growth cones subject to approximately the same local environmental influences.

Barriers to growth cone progress are afforded by the tissues of the tail, such as the processes of connective tissue cells. Such barriers often induce retraction followed by some change of direction of growth cone extension. A varicosity may be left at a site of temporary obstruction.

Cell mitosis, *per se*, either of sheath cells or of fibroblasts, probably has a stimulating effect on nerve sprouts. An active growth cone on touching a small nerve may also induce neuroplasmic movements at the point of contact.

The second, third, and later growth cones usually follow more or less closely the pathway laid down by the first growth cone. Thus, the early unmyelinated nerves are formed. These are soon provided with sheath cells of Schwann, which migrate outward from the central regions and multiply by mitosis. These cells are necessary for the formation of both neurilemma (sheath of Schwann) and the myelin sheath.

The myelin sheath is formed only through the coöperative activity of the nerve fiber and the sheath cell. Not all nerve fibers, however, are equally ripe for myelination. Those which emerge from a myelin sheath (myelin-emergent) are especially ripe for the production of myelin. The differential factor, therefore, responsible for myelin is inherent, not in the sheath cell, but in the nerve fiber.

The myelin is laid down in segments, one segment genetically corresponding to the zone of influence of one sheath cell. The earliest myelin of a segment usually appears near the sheath cell nucleus. It grows by continuous extension in both directions away from the nucleus.

The first segments appear proximally near the nerve roots. Progress of the sheath is in the distal direction, each new segment being added at the end of the myelin line.

Myelination in salamanders usually involves myelin-emergent nerve fibers which are intimately intermingled with non-myelin-emergent fibers. These are often enclosed at first by a single neurilemma. There is a progressive sorting out of such fibers so that the composite nerve containing both myelin-emergent and non-myelin-emergent fibers within a single neurilemma is transformed into a mixed nerve in which some of the constituent fibers are provided with individual sheaths.

Myelin segments, though relatively stable, may undergo various adjustments. Thus, end-to-end anastomosis of two segments may occur; a portion of a segment may be appropriated by the next one and a new node of Ranvier established; bare lengths of nerve fiber between two segments may become ensheathed by the process of intercalation of a whole segment.

Varieties of nerve regeneration and phenomena associated with nerve irritation and recovery in the salamander are quite like those already recorded in the frog tadpole.

Polariscopic observations of all stages of myelinogenesis indicate that the development of anisotropy closely parallels the development of the myelin sheath. The pre-myelin substance of prospective myelin segments is isotropic; the youngest segments are weakly anisotropic; the older and thicker segments are strongly anisotropic.

By means of ciné-photomicrography, permanent records have been obtained which illustrate the essential changes exhibited by individual nerve fibers in salamanders during growth, regeneration, and myelination.

LITERATURE CITED

Speidel, C. C., 1932. Studies of Living Nerves. I. The movements of individual sheath cells and nerve sprouts correlated with the process of myelin-sheath formation in amphibian larvae. *Jour. Exper. Zool.*, **61**: 279.

Speidel, C. C., 1933. Studies of Living Nerves. II. Activities of ameboid growth cones, sheath cells, and myelin segments, as revealed by prolonged observation of individual nerve fibers in frog tadpoles. *Am. Jour. Anat.*, **52**: 1.

Speidel, C. C., 1935. Studies of Living Nerves. III. Phenomena of nerve irritation and recovery, degeneration and repair. *Jour. Comp. Neurol.*, **61**: 1.

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THE GOLGI BODIES IN THE NERVE CELLS OF THE CRAYFISH, CAMBARUS

L. H. KLEINHOLZ

(*From the Biological Laboratories, Harvard University*)

A survey of the literature on the cytology of the arthropod nerve cell with regard to the Golgi apparatus reveals a controversial heterogeneity of opinion. Poluszynski (1911), who worked with the European crayfish, *Astacus,* reported the presence of the Golgi material in the ganglion cells as discrete elements, quite different from the classical reticular pattern of Camillo Golgi, and was confirmed in this by the findings of Bialkowska and Kulikowska (1912), working on insects. Monti (1915), however, questioned these earlier descriptions, having found in her preparations of the nerve cells of *Astacus* that the Golgi apparatus was present as a fine reticulation, a typical and conventional "apparato interno reticulare" of Golgi. Ross (1922) used as material for his studies the large ganglion cells of the American crayfish, *Cambarus,* but could find neither discrete elements nor the typical reticular apparatus. In spite of this, he accepted unquestionably the structures reported by Monti, describing her preparations as the only ones which showed a Golgi apparatus familiar in structure and appearance, and attributing her success to the superb technique at her command.

The results and conclusions of these earlier investigators were not universally and unqualifiedly accepted, for, to add to the already existing confusion there came conflicting reports from various continental workers. Migliavacca (1930) reinvestigated the nerve cell of *Astacus* and reported a typical Golgi apparatus, taking the view that the discrete elements found by former workers were stages in the development of the chondriosomes. Beams and King (1932) used neither *Cambarus* nor *Astacus,* but drew their conclusions from studies on the nerve cord of insects, that the Golgi substance was present in these cells as discrete bodies distributed through the cytoplasm. Another examination of the nerve cells of *Astacus* by Dornesco (1934) revealed the Golgi material as discrete, discontinuous elements.

In spite of the diversity of opinion and contradictory reports on this subject, and the failure of Ross to see any Golgi structure in his preparations, there has been no repetition of Ross' work to see whether the Golgi apparatus was demonstrable and what its appearance would be in the nerve cells of *Cambarus*. It was with this purpose in mind that the present work was undertaken.

I wish here to acknowledge my indebtedness to Professor Alden B. Dawson for much interest and invaluable criticism during the course of this work, and to Dr. John H. Welsh, Jr. for identifying the species of crayfish used.

Two species of crayfish were used in this investigation, *Cambarus virilis* and *Cambarus clarkii*. Only healthy active animals were selected from specimens kept in a concrete aquarium tank. In removing ganglia for fixation, portions of the abdominal cord were rapidly dissected out and dropped into the fixative, with no previous anesthesia or killing of the animal; in some cases the thoracic ganglia were removed for study. The time between exposure of the nerve cord and immersion in the fixative never exceeded one minute, the possibility of postmortem changes in the structure of the nerve cells being thus reduced to a minimum.

The methods used to demonstrate the Golgi apparatus were Ludford's modified Mann-Kopsch technique, the methods of Nassanov-Kolatchev, Cajal, DaFano, and Schridde's process of staining with iron hematoxylin after Champy fixation. The nerve cells proved somewhat refractory to impregnation with osmic acid, the Golgi apparatus not being clearly impregnated until after ten or eleven days of post-osmication at 35° C. in 2 per cent osmic acid. This may explain why Ross was unable to reveal these structures.

The most consistent results were obtained with the Nassanov-Kolatchev technique. Moreover, in several instances, following the routine

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Explanation of Plate I

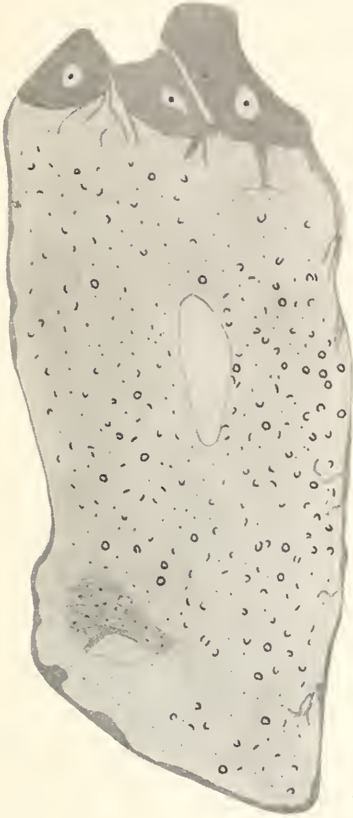
1. Large ganglion cell from abdominal cord of *C. clarkii*, showing mitochondria and discrete Golgi bodies. Champy-Kull fixation and staining.

2. Large ganglion cell from thoracic cord of *C. virilis*, showing distribution of Golgi bodies. DaFano's cobalt nitrate-silver method.

3. Small ganglion cells from abdominal cord of *C. clarkii*, showing mitochondria and Golgi bodies. Champy-Kull technique.

4. Large ganglion cell from abdominal cord of *C. clarkii*, showing Golgi bodies. Champy fixation followed by Schridde's modified mitochondrial stain.

5. Large ganglion cell from thoracic cord of *C. virilis*, showing Golgi bodies The osmic impregnation is heavier in the basal region of the cell. Nassanov-Kolatchev technique.



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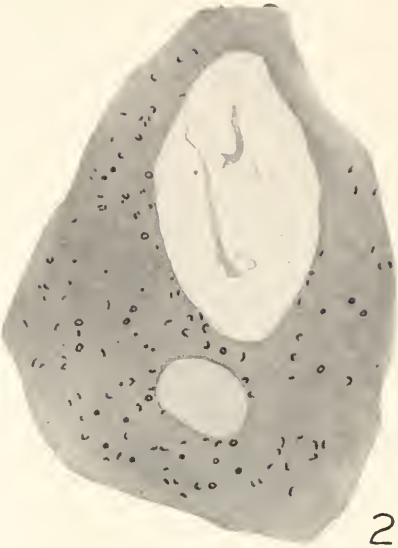

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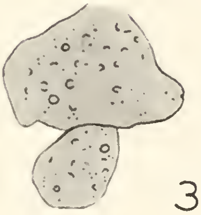

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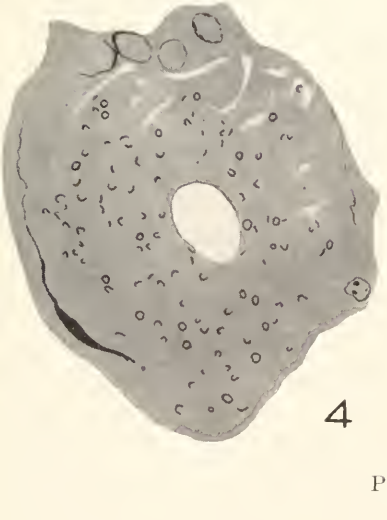

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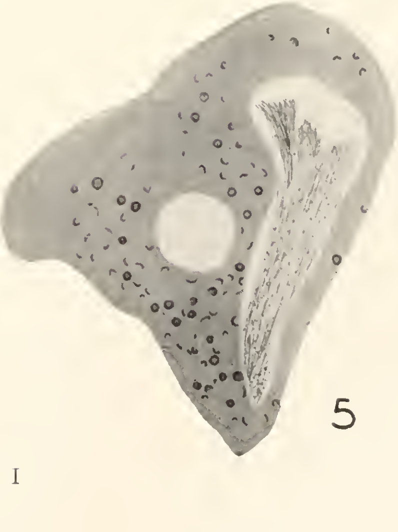

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Plate I

Champy-Kull method for mitochondria, the Golgi bodies were found to have been stained by the acid fuchsin. Fixation in Bouin's picro-formol-acetic acid mixture followed by iron hematoxylin staining gave the negative image of the Golgi structure reported by Beams and King for insect nerve cells. The mitochondria were revealed by fixing and staining according to the method of Champy-Kull.

The degree of impregnation of the osmic acid in the cells varied considerably. In some sections, two cells lying side by side showed differences in the amount of osmication; large cells lying near the periphery of the nerve cord were more heavily impregnated than intermediate and small cells located below the surface; the amount of osmication varied even in the same cell, the peripheral region of the cell being lighter in color than the basal region. Poluszynski attributed this variability to varying physiological states of the cells, and Dornesco attempted to account for a similar phenomenon by explaining it as due to a dilution of the osmic acid by the water content of the cytoplasm. But in the present work, even after renewing the osmic acid daily while the tissues were postosmicating, the same variation in impregnation occurred.

The Golgi bodies are present in the ganglion cells of *Cambarus* as discrete elements. These bodies are plate-like or discoidal in structure with an outer osmiophillic rim and an osmiophobic medulla; in the same cell they are also seen as semi-circles, and curved and straight rods, similar to the structures reported by Poluszynski, Bialkowska and Kulikowska, Beams and King, and Dornesco.

The distribution of the Golgi bodies in the cytoplasm seems to be uniform, with no definite orientation or polarization, though occasional cells were found where they were arranged more or less concentrically about the nucleus.

Nerve ganglia fixed and impregnated by the Cajal and DaFano methods gave good patterns of distribution but inferior morphological pictures in that the Golgi bodies were distorted. Figures 1 and 3 are cells from sections fixed and stained after the Champy-Kull technique. The cells show mitochondria as granules and short rods, and also the Golgi bodies. In no preparation were the mitochondria seen anastomosing to form a reticulum as claimed by Hosselét (1929) for insect nerve cells.

It may be concluded from this paper and from the investigations of others that:

1. There are Golgi bodies present in the ganglion cells of *Cambarus* as discrete elements, now recognized as typical for much invertebrate somatic tissue.
2. They may be readily demonstrated by mitochondrial techniques using acid fuchsin or iron hematoxylin, as well as by the classical osmic acid and silver methods.
3. Ross either did not find the Golgi structures in the nerve cells of *Cambarus* due, possibly, to their refractory behavior to osmic acid, or they were overlooked by him in his preparations.
4. Ross was not justified in accepting the reticular structures reported by Monti for *Astacus* as the typical Golgi apparatus of the crustacean nerve cell.

BIBLIOGRAPHY

Beams, H. W., and R. L. King, 1932. Cytoplasmic Structures in the Ganglion Cells of Certain Orthoptera. *Jour. Morph.*, **53**: 59.

Bialkowska, W., and Z. Kulikowska, 1912. Über den feineren Bau der Nervenzellen bei verschieden Insekten. *Bull. Acad. Sci. de Cracov.*, p. 449.

Dornesco, G. Th., 1934. Recherches sur les constituents cytoplasmiques des cellules nerveuses de Potamobius Astacus L. *Bull. d'Histol. Appliq.*, **11**: 190.

Hosselét, C., 1929. Chondriome à formes d'éléments golgiens dans la cellule nerveuse des Insectes. *Compt. rend. Soc. Biol.*, **100**: 1075.

Migliavacca, A., 1929. Ricerche sulle strutture citoplasmatiche nelle celle nervose e negli elementi neurilemmali. *Arch. Ital. Anat. d. Embriol.*, **27**: 351.

Monti, R., 1915. I condriosomi e gli apparato di Golgi. *Arch. Ital. Anat. d. Embriol.*, **14**: 1.

Poluszynski, G., 1911. Untersuchungen über den Golgi-Kopsch'schen Apparat und einige andere Strukturen in den Ganglienzellen der Crustaceen. *Bull. Acad. Sci. de Cracov*, p. 104.

Ross, L. S., 1922. Cytology of the Large Nerve Cells of the Crayfish (Cambarus), *Jour. Comp. Neur.*, **34**: 37.

STENOSTOMUM PSEUDOACETABULUM (NOV. SPEC.)

JOHN W. NUTTYCOMBE AND AUBREY J. WATERS

(*From the Department of Zoölogy, University of Georgia*)

The species here described fits well into the genus *Stenostomum* as defined by von Graff (1913) but differs sharply from species listed by von Graff (1913) and from those described by Higley (1918), Sonneborn (1930), Kepner and Carter (1931), Nuttycombe (1931), Jones (1932), and Nuttycombe (1932).

Healthy specimens measure from .75 to 2.5 mm. in length. We have never encountered specimens without a fission plane and large individuals frequently show seven well-defined planes (Fig. 1, *F*).

The head is sharply marked off by a constriction just anterior to the mouth and is somewhat spatulate. Posterior to the head the body is cylindrical and tapers gradually into an attenuated caudal region. The posterior end of this region bears a dorsally directed outgrowth (Fig. 1, *A, co*) very similar to that described for *S. rhachiocaudatum*. In *S. pseudoacetabulum*, however, the outgrowth arises at the new fission plane as a postero-dorsal outgrowth from the anterior zoöid (Fig. 1, *D*). In well-fed individuals the body as a whole is very thick and heavy.

In reflected light the animal is white. In transmitted light the integument and anterior region are translucent or pale brownish. The enteric region is gray, brownish, or almost black dependent upon food content. The enteron (Fig. 1, *A, en*) has many glistening vacuoles.

The general ciliary coat is uniform—cilia about as long as epidermis (epidermis other than in free caudal region; here it is thinner than elsewhere) is thick. Non-vibratile cilia, three to four times as long as those in general coat, have been observed in the preoral region but not elsewhere.

Rod-shaped rhabdites (Fig. 1, *E*) vertically disposed, and uniformly distributed lie just beneath the surface of the epidermal cells.

The two ciliated pits (Fig. 1, *A, cp*) are of medium size and laterally placed.

The cephalic ganglia (Fig. 1, *A, cg*) conform to the general pattern for the genus. Light-refracting bodies associated with the ganglia have not been observed.

From above the mouth at rest resembles very strongly a trematode acetabulum. This feature suggested the name *pseudoacetabulum*. This

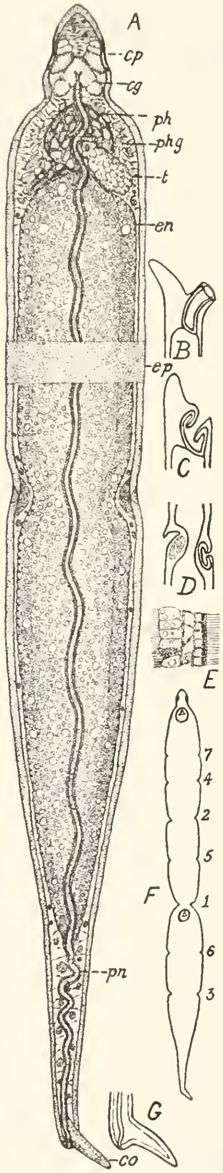

FIG. 1. *A, S. pseudoacetabulum,* dorsal aspect in optical section; *cp,* ciliated pits; *cg,* cerebral ganglia; *ph,* pharynx; *en,* enteron; *phg,* pharyngeal glands; *t,* testis; *pn,* protonephridium; *ep,* dorsal view of epidermis only; *co,* caudal outgrowth. *B,* diagrammatic sketch of anterior region with pharynx everted. *C,* diagrammatic sketch of anterior region with pharynx inverted. *D,* fission plane with developing pharynx and developing caudal outgrowth. *E,* details of body wall and enteron to show endoderm, pseudocoel, and ectoderm. *F,* outline of chain of eight zoöids labelled to indicate relative age of fission planes. *G,* lateral view of caudal outgrowth with terminal protonephridial opening.

sucker-like appearance is due to the fact that the lips are invaginated so deeply as to involve about the anterior one-third of the pharyngeal wall (Fig. 1, *C*). This region may be everted rapidly to form a proboscis-like structure (Fig. 1, *B*).

The pharynx is widened posteriorly to form a cap over the anterior end of enteron. The pharyngeal walls are highly muscular and prominently glandular (Fig. 1, *A, phg*). A sphincter occurs between the pharynx and the enteron. The pharyngeal lumen is ciliated. The radial muscle fibers from the pharyngeal wall to the body wall are very numerous.

The enteron (Fig. 1, *A, en*) conforms in shape to the body region in which it lies. It terminates considerably anterior to the caudal end of the animal and leaves an enteron-free caudal region. The enteric epithelium is approximately twice as thick as that of the epidermis (Fig. 1, *E*). Its cells are prominently vacuolate and finely ciliated. Occasional granular gland cells occur.

The general mesenchyme is restricted to the pseudocœl and the spaces between the organs. Its cells are of various shapes and sizes.

The protonephridium (Fig. 1, *A, pn*) passes posteriorly from the preganglionic region beneath the transverse commissure of the cephalic ganglia, dorsal to the pharynx and enteron and empties terminally beneath the dorsal outgrowth at the posterior end.

About half of the specimens studied were sexually mature as males. The testis (Fig. 1, *A, t*) is located above and slightly to the right side of the pharynx.

This species was collected in large numbers from ponds in Bulloch County, Georgia.

Specific diagnosis: Stenostomum pseudoacetabulum differs from all other species of the genus in that its lips, at rest (Fig. 1, *C*), are inverted well into the pharynx. Furthermore, it differs from all other species, except *S. rhachiocaudatum*, in having a single, dorsally-directed, caudal outgrowth. *S. pseudoacetabulum,* however, differs sharply from *S. rhachiocaudatum* in that it has no light-refracting bodies, no vesicular bodies in the epidermis and a terminal opening for the nephridiopore, whereas *S. rhachiocaudatum* has paired light-refracting bodies, prominent vesicular, epidermal bodies and the opening of the nephridiopore is not terminal.

LITERATURE CITED

Graff, L. von, 1913. Turbellaria. II. Rhabdocœlida. Berlin.

Higley, Ruth, 1918. Morphology and Biology of Some Turbellaria from the Mississippi Basin. Biol. Monographs, Univ. of Ill., Urbana, Ill.

Jones, E. Ruffin, Jr., 1932. Stenostomum carnivorum n. sp. *Zool. Anzeig.*, **97**: 292.

Kepner, W. A., and J. S. Carter, 1931. Ten Well-defined New Species of Stenostomum. *Zool. Anzeig.*, **93**: 108.

Nuttycombe, J. W., 1931. Two New Species of Stenostomum from the Southeastern U. S. *Zool. Anzeig.*, **97**: 80.

Nuttycombe, J. W., 1932. Two New Species of Stenostomum. *Zool. Anzeig.*, **101**: 29.

Sonneborn, T. M., 1930. Genetic Studies on Stenostomum incaudatum (Nov. Spec.). *Jour. Exper. Zool.*, **57**: 57.

THE EFFECT OF CENTRIFUGING ON THE POLARITY OF AN ALGA, GRIFFITHSIA BORNETIANA

VICTOR SCHECHTER

(From the Department of Biology, College of the City of New York, and the Marine Biological Laboratory, Woods Hole, Mass.)

INTRODUCTION

The nature of differentiation and particularly the causes of the axial differentiations which define the polarity of an organism, are problems which have been attacked from many different angles. Experimental procedures for a long time centered about the concept of specific organ-forming substances. At first the simple expedient of overturning fragments was utilized to observe the effect of heavier and lighter substances upon regeneration through the action of gravity. This method, employed chiefly with plant material, showed that although orientive adjustments occurred, there was usually little effect upon the actual point of origin of new roots and shoots (e.g. Vöchting, 1885, 1906; Loeb, 1924). Exceptional cases, perhaps most clearly illustrated in the lower plants (Noll, 1900; Wulff, 1910) were explained, often by the authors themselves, as due to the stimulation of preformed centers by accumulated indifferent nutrients.

At Naples, in 1901, Morgan attempted to analyze the effect of gravity on the regeneration of Antennularia through the use of a "rotating wheel." The centrifuge method, initiated by Lyon in 1906 and adopted by many others,[1] may have developed from this simple device. As with gravity, however, it was shown clearly that centrifugal displacement of visible granules is ineffective in altering the normal pattern of growth, except of course in the event of injury. Lillie (1909) therefore concluded that polarity is a property of the ground substance; and Conklin (1931) decided that the effect of extremely high speeds (upon the development of ascidian eggs) is due to the displacement of specific areas in the cytoplasm.

The case which I wish to present in this paper has several points of interest. It is the only one I know of in which a new axis of polarity, involving normal organ formation, is set up by centrifugal force. The results are obtained at surprisingly low speeds, which renders improbable the displacement of cytoplasmic areas. The facts are of interest, also, because of the light which they may be construed to throw upon the doctrine of "specific stuffs."

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[1] Morgan and Lyon, 1907; McClendon, 1909; F. R. Lillie, 1909; Morgan, 1908, 1909, and 1910; Morgan and Spooner, 1909; Boveri, 1910; Conklin, 1931 and previously.

Material and Method

During the course of an investigation on the effect of direct electric current upon regeneration in the alga, *Griffithsia bornetiana* (Schechter, 1934), electrophoresis of chromatophores to the position where rhizoids arose was frequently observed. Preliminary experiments with the centrifuge to determine whether there was any causal relationship between these two phenomena gave negative results, but indicated an interesting effect upon the shoots. The present report is concerned with this effect. The experimental work was done chiefly during the summer of 1934, and the substance of the results presented at the Marine Biological Laboratory at the seminar of August 7, 1934. I wish to acknowledge gratefully helpful conferences with Dr. L. G. Barth.

Freshly collected tufts of the alga were cut into suitably sized fragments and centrifuged continuously for about 24 hours with a force of approximately 150 × gravity. Stratification of the cell contents began in about an hour and after 24 hours the chromatophores were accumulated as a dense cap of material at the centrifugal pole, sharply marked off from the rest of the cell which had become quite transparent. The fragments were then allowed to develop in Syracuse dishes of sea water until new shoots appeared. An ocular micrometer was used for measuring and sketches were made with the aid of a camera lucida.

In the early experiments there was much damage to the material, arising toward the end of the period of centrifuging. By avoiding overcrowding and carrying out the experiments at 19° C. (7–9° below room temperature during the hot weeks of 1934), injury was much reduced and often almost entirely avoided. Under carefully controlled conditions one batch of material was centrifuged continuously for a period of four days with a force of 61 × gravity. Shoots and rhizoids were regenerated.

After the usual amount of centrifuging (about 150 × gravity for 24 hours) it required a week or more before redistribution of the cell contents restored the normal appearance. This unusual duration of stratification may be of significance with respect to the effect upon polarity.

RESULTS

Out of 18 experiments the material died in three, probably due to high temperature, and in 12 of the remaining 15 there was more or less determination of shoot origin in each, as described below:

When the cell apexes were oriented outward from the axis of rotation shoots always appeared in normal positions (Fig. 1, sketches 12, 13, 14). On the other hand, as shown in sketches 1, 2, 6, 8 and Photographs 4 through 12, shoots often arose from the base when the cell bases were centrifugally oriented. However, basal shoot formation did not necessarily exclude proliferation from the apexes of the same cells (sketches 3–5, 7, 9–11). It is to be noted that all of the shoots were quite normal in appearance. For comparison several young shoots on control material are shown in Photograph 1. Photographs 2 and 3 are of basally centrifuged cells in which the normal polarity nevertheless remained unaltered.

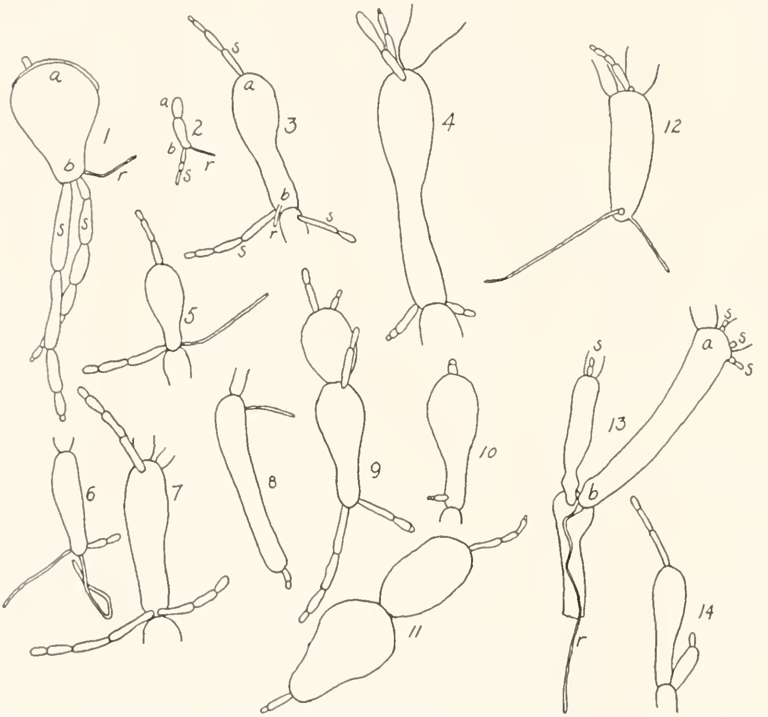
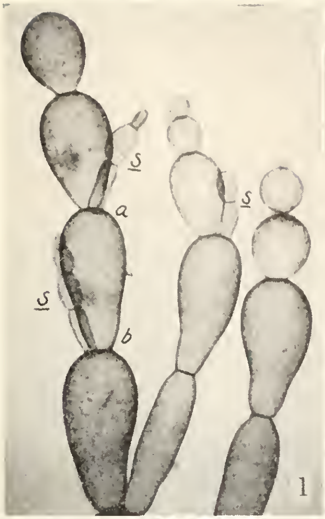


FIG. 1. Camera lucida sketches of experimental material.

Sketches 1–11 of material centrifuged basally. Induced basal, and some shoots in normal position, are shown on the same or different cells.

Sketches 12–14 of material centrifuged apically. The normal polarity was retained. (*a*—cell apex, *b*—base, *s*—new shoot, *r*—rhizoid.)



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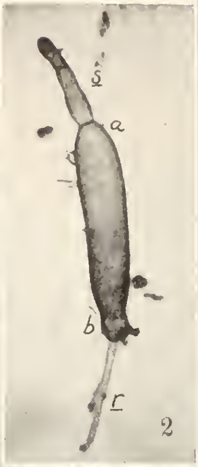

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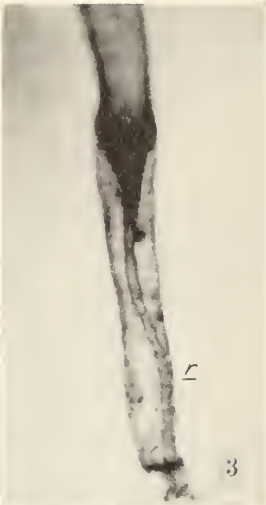

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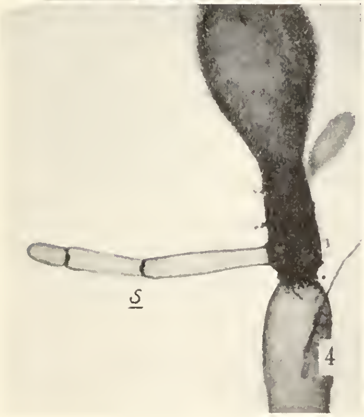

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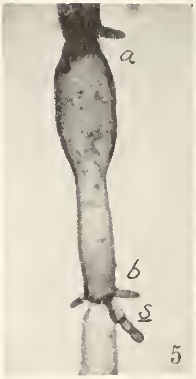

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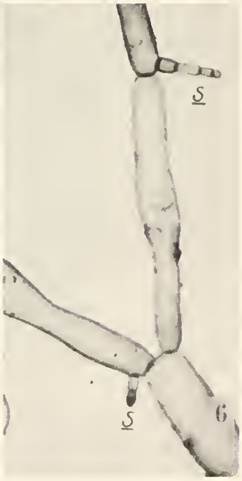

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EXPLANATION OF PLATES

Plate I

Plates I and II contain photographs of control (Photograph 1) and of experimental material centrifuged basally.

1. Control showing two-, three- and four-celled new shoots in normal apical position.

2. A cell, centrifuged basally, in which the original polarity was retained.

3. The base of a cell with material accumulated during centrifugation. Original polarity was retained in this case as shown by rhizoid in normal position.

4, 5, 6. Baso-lateral shoots following centrifugation. The cells were still part of the original filament, where they occupied a position about midway between apex and base of the plant.

An inspection of the angle of induced shoots upon isolated cells and upon those cells still part of a filament, reveals an interesting situation. The new shoots came off laterally (Photographs 4, 5, 6) when upon cells forming part of a filament. On free cells they usually arose directly from the base (Photographs 8, 10, 11, 12); or there was occasionally a shoot in each position. Photograph 7 shows a male apical cell partially dislodged from the chain with both a lateral and an almost directly basal shoot. In Photograph 9, where the adjoining cell is dead, the basal shoot has grown directly through the dead region. These observations indicate a tendency toward a rather local effect directly in line with the axis of centrifuging.

Unlike shoot formation, rhizoid origin from centrifuged cells was not materially affected. In fact rhizoids often appeared on the cell base together with induced shoots (Photograph 11). In Photograph 12 a new shoot, by forming a rhizoid on its own axis, has completely established independent polarity.

The series of photographs demonstrates also that shoot-forming polarity may be reversed in cells anywhere along the axis of the alga, from the extreme apex to the extreme base.

Data concerned with the frequency with which reversals occur is based mainly upon four experiments. In cells oriented so that heavier materials were thrown toward the apex 229 apical shoots were formed. When oriented in the opposite direction a roughly equivalent number of cells produced 214 apical and 121 basal shoots. The centrifugal forces in these experiments were 100 to 220 × gravity, an average of 160 ×.

It seems interesting that the new shoots were generally smaller in the experiments than in the controls. In one case, for example, 14 typical shoots upon basally centrifuged cells averaged 2.6 cells in number (range 2–5) and 0.75 mm. in length. Where the heavier substances were thrown into the cell apexes 17 shoots, varying from 1 to 6 cells, averaged 3.3 cells and 1.17 mm. in length. Eleven typical shoots upon control material were 3.0 mm. in average length and consisted of from 2 to 9 cells, an average of 6.5. Perhaps correlated with their smaller size is the general observation that new shoots occurred more frequently on centrifuged cells. No exact data on this point are available at present.

These measurements, besides showing a difference between shoots on centrifuged and non-centrifuged cells, also bring out the fact that orientation of the cells during centrifugation affected the size of new shoots. A summation of the data of several experiments indicates that the difference is a general one. One hundred and four apical shoots (unaffected in origin) upon basally centrifuged material averaged 2.9 cells and 0.9 mm. in length; whereas 41 basal shoots consisted of 2.54

cells and were 0.78 mm. long. Also supporting are measurements made in one experiment upon 15 basally centrifuged cells each of which formed both apical and basal shoots, as in sketches 5 and 10, Fig. 1. The average length of the former was 0.85 mm. and of the latter 0.76 mm.

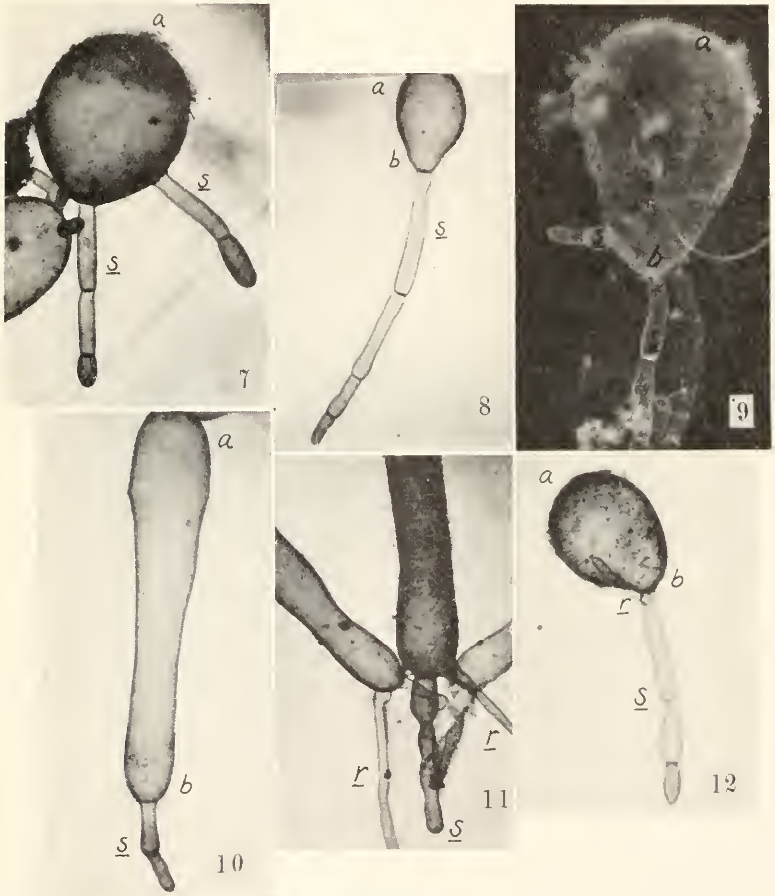

Plate II

7. An apical cell partially dislodged from the filament. One new shoot formed laterally, one almost directly basally.

8. An isolated apical cell. New shoot directly basal.

9. An apical cell held to the filament by a dead adjoining cell. A basolateral shoot and a basal shoot were formed.

10. A basal cell. Basal shoot on the free basal end.

11. Basal cells. Induced basal shoot and normal basal rhizoids are both present.

12. A basal shoot from an apical cell. The basal cell of the new shoot formed a rhizoid normal to the polarity of the induced shoot.

In all of the instances cited above the basal (induced) shoots were smaller than the apical. If we may assume a fairly regular rate of growth, then the size of shoots is an indication of the time at which they began to form and the difference in size should be a measure of the amount of centrifuging required to induce basal shoot formation.

Discussion

In view of numerous unsuccessful attempts to control polarity by centrifuging and the absence of reports in this direction incidental to the large amount of other work involving the same technique, the results presented in this paper appear to comprise the only case in which a normal determinative effect upon development has been obtained. It is therefore of particular importance to investigate the underlying mechanism of centrifuge action in this material. To be sure, the syncytial nature of the *Griffithsia* cell (see Lewis, 1909) offers a different type of biological system than do animal eggs and embryos, upon which practically all other work involving centrifugation has been done. It may be that some feature of organization peculiar to a syncytium makes the results possible. But in nature *Griffithsia* shoots are formed on the cell apexes regardless of orientation with respect to gravity. We would have to concede to the centrifuge, therefore, a greater efficiency and perhaps a higher specificity in the separation of cellular elements than can be attributed to gravity.

The effect obtained by centrifuging *Griffithsia* also seems, in other respects, somehow unrelated to the action of gravity. Loeb (1894) found that hydranths of Antennularia were regenerated from the upper ends of overturned stems. With plants, where it has been possible to obtain responses other than those of orientation, results have been similar. In all of these cases the new growths formed at the upper ends were those normally found in that position in nature. Quite on the contrary with *Griffithsia;* after centrifuging the new shoots were produced where the heavier materials had been thrown, a position synonymous with the lower end of the organism in the field of gravity; one normally associated with rhizoid formation.

The possibility therefore arises that the effect is due, not to the movement of shoot-forming substances, but to a stimulation set up by an unusual concentration of rather non-specific materials. The simultaneous appearance of shoots on both poles of some of the cells also weighs against the existence of a definite shoot-forming substance, necessarily limited in amount, and is in accord with the above hypothesis. An analogy can perhaps be drawn with activation of unfertilized eggs by various chemical and physical agencies.

May I, in concluding, take this opportunity to acknowledge my indebtedness for help and stimulation to Dr. Charles F. Hunt, whose understanding interest extended from medicine to other fields of science, and whose recent death has removed a rare and valued friend.

SUMMARY

Normal shoots appear upon *Griffithsia* cells at the point where heavier substances are concentrated by prolonged low speed centrifuging. In this way reversal of polarity may be produced anywhere along the plant axis. The possibility is suggested that the centrifuged substances are not directly determinative but act by stimulation.

LITERATURE CITED

BOVERI, TH., 1910. Über die Teilung Centrifugierter Eier von Ascaris megalocephala. *Arch. Entw.-mech.*, **30**: 101.

CONKLIN, E. G., 1931. The Development of Centrifuged Eggs of Ascidians. *Jour. Exper. Zool.*, **60**: 1.

LEWIS, I. F., 1909. Life History of Griffithsia bornetiana. *Ann. Bot.*, **23**: 639.

LILLIE, F. R., 1909. Polarity and Bilaterality of the Annelid Egg. Experiments with centrifugal force. *Biol. Bull.*, **16**: 54.

LOEB, J., 1894. On Some Facts and Principles of Physiological Morphology. Biological Lectures at the Marine Biological Laboratory in 1893. Ginn and Co., Boston.

LOEB, J., 1924. Regeneration from a Physicochemical Viewpoint. New York.

LYON, E. P., 1906. Some Results of Centrifugalizing the Eggs of Arbacia. *Am. Jour. Physiol.*, **15**: xxi (Proc. Am. Physiol. Soc.).

MCCLENDON, J. F., 1909. Cytological and Chemical Studies of Centrifuged Frog Eggs. *Arch. Entw.-mech.*, **27**: 247.

MORGAN, T. H., 1901. The Factors that Determine Regeneration in Antennularia. *Biol. Bull.*, **2**: 301.

MORGAN, T. H., 1908. The Effects of a Centrifugal Force on the Eggs of Cumingia. *Science*, **27**: 446.

MORGAN, T. H., 1909. The Effects Produced by Centrifuging Eggs Before and During Development. *Anat. Rec.*, **3**: 155.

MORGAN, T. H., 1910. Cytological Studies of Centrifuged Eggs. *Jour. Exper. Zool.*, **9**: 593.

MORGAN, T. H., AND E. P. LYON, 1907. The Relation of the Substances of the Egg, Separated by a Strong Centrifugal Force, to the Location of the Embryo. *Arch. Entw.-mech.*, **24**: 147.

MORGAN, T. H., AND G. B. SPOONER, 1909. The Polarity of the Centrifuged Egg. *Arch. Entw.-mech.*, **28**: 104.

NOLL, F., 1900. Über die Umkehrungsversuche mit Bryopsis. *Ber. d. Deut. Bot. Gesell.*, **18**: 444.

SCHECHTER, V., 1934. Electrical Control of Rhizoid Formation in the Red Alga, Griffithsia bornetiana. *Jour. Gen. Physiol.*, **18**: 1.

VÖCHTING, H., 1885. Über die Regeneration der Marchantieen. *Jahrb. f. wissensch. Botanik*, **16**: 367.

VÖCHTING, H., 1906. Über Regeneration und Polarität bei höheron Pflanzen. *Bot. Zeitung*, **64**: 101.

WULFF, E., 1910. Über Heteromorphose bei Dascycladus clavæformis. *Ber. d. Deut. Bot. Gesell.*, **28**: 264.

CHANGE IN SIZE AND SHAPE OF AGEING EGGS (ARBACIA PUNCTULATA)

A. J. GOLDFORB

With Technical Assistance of Victor Schechter and
Milton Landowne

(From the College of the City of New York)

Using the method of Lucké and McCutcheon (1926, 1927, 1931), we found that the permeability of unfertilized eggs (*Arbacia punctulata*) increased progressively with age.[1] The factor in their formula that gave us considerable trouble was size of eggs prior to testing. Size may be determined by measuring each egg before as well as during the test. But this is impracticable. Size is usually determined by extrapolation. But several extrapolated values may frequently be obtained, and the resulting permeability rates may be significantly different. Size may be determined by measuring control eggs at successive ages under the same conditions. When eggs were so measured we found that a cyclical change in size occurred with age.

PROCEDURE

Eggs were measured during the summers of 1930, 1931, 1932, and 1933. The *Arbacia* were received directly from the collecting boat. Those females were chosen whose eggs were in good physiological condition, and which had the largest number of spherical eggs. The eggs of each female were strained and washed in 200 cc. sea water, divided into two or more portions, kept in flat finger bowls, at 16.5 to 22.0° C. The temperature at successive ages varied by 1 to 3° C. The supernatant sea water was changed twice daily. Sea water collected at or near high tide was filtered and stored before use. The pH was 8.3.

The diameters of 50 consecutive *spherical* eggs were measured with an ocular micrometer at 660 ×, or with a filar micrometer at 260 ×. Measurements are believed accurate within $\pm 0.4 \mu$. The probable error is $\pm 0.11 \mu$.

VARIATION IN SIZE OF EGGS FROM DIFFERENT FEMALES

Table I gives, for 25 females, the number of eggs measured, their age and physiological condition, the average and extreme sizes. One thousand and fifty eggs, in good physiological condition, $\frac{1}{4}$ to 5 hours old, were measured. The average size of eggs from different females varied widely, viz., by 36.9 per cent.

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[1] In press.

In previous investigations, age and physiological condition have usually not been given, or diminutive (erupted) as well as oversized (dead or fused) eggs have undoubtedly been included in the data (Glaser, 1914*a*). It is also probable that the average size was based on too few eggs (Glaser, 1924; Goldforb, 1917*b*). Lillie (1916) measured 58 eggs, presumably from one female, whose average size was 213,000 μ^3. Lucké, McCutcheon, and Hartline (1931) measured 450 eggs, in good physiological condition, in groups of 50 or 100, from 6 females. The average sizes varied from 182,300 to 224,500 μ^3, mean 201,183 μ^3. E. N. Harvey (1932) quotes another series of measure-

TABLE I

Variation in Size of Freshly-shed Eggs from Different Females

Series	Date	No. of Eggs	Age	Range in Volume	Av. Volume	Cleavage
			hours	$\mu^3 \times 100$	$\mu^3 \times 100$	*per cent*
I_2	7/ 2/32	40	1¾	2262–2514	2431	100
E_2	6/28/32	50	½	2157–2544	2344	—
G_2	7/ 1/32	14	2	2105–2372	2228	—
B	6/30/31	28	½	1957–2276	2128	99
I_3	7/12/33	50	1½	2018–2211	2125	99
F_2	7/18/32	50	5	2029–2236	2120	—
J_3	7/17/33	55	3⅓	2039–2189	2111	100
$P_{3.3}$	7/31/33	50	4½	1957–2167	2056	100
V	7/29/30	22	¼	1979–2156	2050	—
N	7/14/32	49	4	1962–2103	2049	100
$P_{3.1}$	7/31/33	50	5	1957–2167	2048	100
W	8/ 5/30	40	½	1936–2166	2005	—
G_1	7/21/31	50	1½	1800–2146	1999	93
E_1	6/28/32	50	½	1857–2145	1999	—
$P_{3.2}$	7/31/33	50	5½	1937–2103	1998	100
J_1	8/ 3/31	50	3	1938–2165	1996	99
P_0	7/15/30	9	1½	1858–2139	1979	—
U	7/25/30	12	3	1798–2086	1963	—
F_1	7/18/31	50	1	1761–2082	1959	100
I_1	7/27/31	50	4	1781–1917	1883	100
H	7/11/33	50	3	1762–2060	1909	100
L	7/ 6/33	50	½	1788–1954	1873	99
G_3	7/ 7/33	60	4	1669–1998	1848	100
P_2	7/26/32	30	5	1698–1930	1783	100
K	8/ 6/31	50	3	1724–1897	1776	100
Total 25		1050			Av. 202,640 μ^3	
Variation					36.9%	

ments by these authors, viz., 550 eggs from 4 females, average size 212,200 μ^3. E. B. Harvey (1932) records an average of 203,690 μ^3 (number of eggs and females not given). In our series, Table I, the averages ranged from 177,600 to 243,100 μ^3, mean size 202,640μ^3.

The variation in size of eggs from any female is small. The curve of distribution is quite narrow. For the sake of brevity, only the extreme sizes are given in Table I. These extremes varied from 7.2 to 19.7 per cent, average 12.6 per cent. It should be noted that the curve of distribution may be asymmetrical. For example, in Series I only 6 per cent, while in Series G_1, 62 per cent of the eggs were larger than the mode. When the averages were similar, the range may be different. For example, in Series G_1, E_1, and J_1, the average sizes were 199,900, 199,900, and 199,600 μ^3 respectively; the minimal sizes were, however, 180,000, 185,700, and 193,800 μ^3 respectively.

Table I also suggests that size may decrease as the breeding season advances.

The wide variation in size of eggs from different females (Table I) is not due to physiological deterioration, for 99 to 100 per cent cleaved normally, or 95 or more per cent formed parthenogenetic membranes in distilled water; nor is this variation due to age, since eggs of the same age show similar variation in size.

The average of 50 consecutive spherical eggs is not as accurate an index of size as desired. Nevertheless, it is believed that Table I affords a more adequate picture than has heretofore been offered of the large variability in size of freshly-shed eggs of known age and physiological condition from different females, and of the smaller variation in size of eggs from any one female.

It may be noted that eggs from different females differ widely not only in size but in fertilization membrane formation (Goldforb, 1917*a*), cleavage (Goldforb, 1917*b*), and agglutinin (Goldforb, 1929*a*).

Increasing Size During Early Ages

When samples were measured at successive ages there was a progressive *increase* in size during the first 23 to 50 hours.

Series *W*, Table II, illustrates a medium increase. The smallest diameters, between ½ and 24½ hours, increased from 71.67 to 72.53 μ. The largest diameters increased from 74.39 to 75.10 μ. The number of large eggs also increased significantly with age. The average size increased progressively from 200,500 to 207,200 μ^3. The increase during the first 44 hours was 3.07 per cent.

Series *L* illustrates a small increase. The smallest diameters were the same size between ½ and 29½ hours. The maximum diameters increased slightly, i.e., from 72.0 to 72.6 μ. The number of large eggs increased, between ½ and 8½ hours. The average size increased from 187,300 to 190,800 μ^3, or 1.9 per cent.

Thirteen series are summarized in Table III. The increases in size for the given ages were 1.1, 1.9, 2.1, 2.2, 2.3, 3.0, 3.2, 3.3, 4.7, 5.6, and 5.8 per cent respectively. Table III gives the minimum and maximum

TABLE II

Variation in Size of Eggs with Age

Series	Age	No. of Eggs	Percentage of Eggs							Range in Diameter	Av. Size	Increase or Decrease	Cleavage
			Below 28.0	28.1 to 28.5	28.6 to 29.0	29.1 to 29.5	29.6 to 30.0	30.1 to 30.5	Above 30.5				
			Ocular Units Diameter										
	hours									μ	$\mu^3 \times 100$	*per cent*	*per cent*
W	½	40	0	10	60	17	12	0	0	71.67–74.39	2,005		
	3½	40	0	10	50	27	10	3	0	71.67–74.65	2,018		
	6½	40	0	4	47	35	13	1	0	71.92–74.65	2,025		
	18	40	0	2	45	25	28	0	0	71.92–74.39	2,043		
	24½	40	0	0	55	30	14	1	0	72.53–74.65	2,035		
	30½	40	0	5	57	5	18	3	7	71.67–75.10	2,060		
	44	20	0	0	30	55	15	0	0	71.92–74.91	2,072	+3.0	
	48	20	20	15	55	10	0	0	0	69.43–73.17	1,914	−7.6	
L	½	50	0	46	52	2				69.90–72.00	1,873		99
	3½	50	0	32	56	12				69.90–72.60	1,897		96
	8½	50	0	20	68	12				69.90–72.60	1,908	+1.9	96
	15½	50	0	52	44	4				69.90–72.60	1,890		89
	29½	50	0	50	44	6				69.90–72.60	1,866		
	37½	50	20	64	16	0				68.70–72.00	1,800		
	47½	50	6	64	30	0				69.20–71.91	1,832	−4.2	73

diameters at the initial age and at the age when eggs were largest. It will be noted that the minimum diameters increased with age in most series; the maximum in all but one series. The abbreviated table does not, however, show the progressive increase in numbers of large eggs. Table III shows an increase of 2,400 μ^3 to 11,500 μ^3. For reasons to be given later, the increase with age is believed to be even larger.

It is therefore concluded that, under the stated conditions, a significant increase in size of control eggs occurred as the eggs aged; that the increase may be measurable by the third hour, may continue for 22 to 50 hours, and may be as much as 5.8 per cent.

DECREASING SIZE DURING LATE AGES

In every series in which eggs were measured at late and frequent ages there was a much greater change in size but in the reverse direction. In Series *W* (Table II) the minimum diameter decreased from 71.92 to 69.43 µ. The maximum diameter decreased from 75.10 to 73.17 µ. The curve of distribution shifted sharply towards the left. The average size decreased 7.6 per cent. In Series *L* there was a slower decrease of 4.2 per cent.

TABLE III

Summary of Thirteen Series Showing Cyclical Change in Size with Age

Series	Ages Measured	Increase		Late Decrease	Diameter			
					Initial Age		Intermediate Age	
					Minimum	Maximum	Minimum	Maximum
	hours	μ^3	_per cent_	_per cent_	µ	µ	µ	µ
P_0	1, 2, 3, 20, _23,_* 27, 30, 43, 54	11,500	5.8	4.6	70.80	74.21	71.82	75.22
U	3, 5, 7, 9, _24,_* 27, 30	9,200	4.7	†	70.20	73.58	70.71	75.40
V	1/10, 2, 5, 8, 21, 28, _34_*	11,500	5.6	9.7	72.21	74.29	72.21	77.90
W	1/2, 3, 6, 18, 24, 30, _44,_* 48	6,200	3.0	7.6	71.67	74.39	71.92	74.91
B	1/2, 2, 5, 10, _23,_* 28, 32, 48, 52, 56, 72	2,400	1.1	9.5	72.04	75.76	73.28	76.01
F_1	1, 8, 24, 31, _47,_* 55	4,300	2.2	0.4	69.43	73.40	69.92	74.39
G_1	1, 8, 24, 31, _50_*	6,300	3.2	—	69.93	73.90	71.92	74.39
I_1	4, 42	0	0	1.8	69.80	71.53	69.45	72.15
J_1	3, _31_*	4,500	2.3	†	71.67	74.39	71.92	74.63
K	3, _25_*	3,700	2.1	†	68.94	71.18	69.43	71.68
I_2	2, 6	600	0.2	†	75.60	78.30	76.50	78.60
L	1/2, 3, _9,_* 15, 29, 37, 47	3,500	1.9	4.2	69.90	72.00	69.90	72.60
P_2	5, _22_*	5,800	3.3	†	68.70	71.70	69.00	71.70

* Indicates ages when maximal size was observed.

† Eggs not measured at late ages.

Table III shows a decrease during late ages of 4.6 per cent (Series *Po*), 9.5 per cent (Series *B*), and 9.7 per cent (Series *V*).

COMMENTS ON CYCLICAL CHANGE IN SIZE

With age, eggs enlarged and the curve of distribution shifted to the right. At intermediate ages some eggs continued to swell, while others, having reached their maximum size, progressively diminished in size. The curve became bimodal. The condensed tables do not adequately show this phenomenon. At later ages all the eggs have shrunken and a monomodal curve is reëstablished, but is now shifted sharply to the left.

Eggs swelled more than indicated in the tables. Since the initial size was measured $1/10$ to 5 hours after shedding, the later the initial age, the greater had been the swelling. Hence the increase based upon this late initial size is correspondingly too small. It is also improbable that the eggs were measured exactly when they reached their maximum size. If measured before or after this maximum, the increase would be correspondingly too small.

At late ages, eggs tend to fuse into giant eggs (Goldforb, 1913). Upon death, intact eggs swell considerably. Many eggs cytolyze with closely adhering fragments. All these eggs were excluded. The increased size discussed above refers to a change in living eggs, which were able to cleave and were morphologically indistinguishable, except for color and granulation, from freshly-shed eggs.

Increase in size with age may not be attributed to greater flattening against the bottom of the dish. McCutcheon, Lucké and Hartline (1931) have demonstrated that flattening does not occur in freshly-shed *Arbacia* eggs. We believe that flattening did not occur during the first ca. 25 hours. During late ages, however, flattening probably did take place, for the viscosity of the egg progressively decreased and the egg membrane softened considerably. Yet in spite of this probable flattening and pseudo-enlargement, during late ages, the eggs progressively decreased in size.

The change in size may not be attributed to changes in H ion concentration of the medium. The supernatant sea water was progressively more acid. The pH changed from 8.3 to 7.9. But this degree of change is too small (Lucké, McCutcheon, 1926), and does not account for the cyclical change in size.

As shown elsewhere,[2] permeability to water increased progressively and markedly with ageing of eggs. It is this increased water intake which we believe to be one of the major factors in the increasing size of ageing eggs.

The decreasing size during late ages may be accounted for on the basis of the following observations. Beginning about 23 hours after shedding, the plasma membrane ruptured and a small pellicle, about $1\frac{1}{2}\ \mu$ diameter, was ejected. The pellicle, containing minute grey granules and fluid, rounded out quickly and immediately formed a membrane. The ruptured membrane closed completely in 1 to $1\frac{1}{2}$ minutes. Within 2 minutes the egg, except for adhering pellicle, appeared normal and when fertilized cleaved normally.

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[2] In press.

At later ages (about 30 to 40 hours) a larger cone formed. The membrane burst at or near the apex of the cone. The pellicles were larger (i.e. to 3.75 μ diameter) and contained small and large granules. The egg membrane reformed more slowly (2 to 3 minutes). The egg near the erupted area remained pale for several minutes. Later (50 or more hours) more eggs ruptured, the pellicles were larger and formed in succession. At this time pellicles graded into the larger globules characteristic of cytolyzed eggs.

The smallest pellicles, not including water discharged into the medium, were estimated as ca. 1 μ^3. With age, their size increased to and beyond 27.5 μ^3. If the discharged water be included, the mass of ejected materials is correspondingly greater. It is this repeated ejection of substances that is held responsible in largest part for the decreasing size of the eggs during late ages.

TABLE IV

Showing Variation in Percentage of Ellipsoid Eggs, Freshly-Shed, 16 Females

Series	B_1	L_1	E_1	E_3	E_2	G_0	G_1	B_2	L_2	I	J	K	N	F	P	B_3
Ellipsoid eggs, %	62	42	26	32	45	10+	50	40	20	35	37	15	18	29	35	36
No. of eggs examined	74	50	68	72	74	50	28	—	50	62	50	50	49	70	46	50
Age in hours	½	½	½	½	½	2	2	2	2	2	3	3	4	5	5	5
Cleavage (per cent)	99	97	*	*	*	93	*	*	99	100	99	100	100	*	100	*

* In distilled water formed 95 per cent or more parthenogenetic membranes.

Not only does size change cyclically but the rate of fertilization, of cleavage, and of sperm agglutination (Goldforb, 1918, 1929*a* and *b*) also show a similar cyclical phenomenon, with age.

It has been suggested that in calculating the permeability rate, the size of eggs at all ages be averaged; but from the above discussion it is evident that any constant size is contrary to the facts, and since a small error in size may considerably alter the permeability rate, a much more accurate rate may be obtained only when the cyclical trend and the size of eggs at each age are known.

CHANGE IN SHAPE OF AGEING EGGS

Variation in Percentage of Globular Eggs from Different Females

The percentage of globular eggs in different females varied widely. Table IV summarizes the results in 16 series. Eggs from 5 females examined within ½ hour after shedding contained 62, 45, 42, 32 and

26 per cent ellipsoid eggs respectively. In 5 females, whose eggs were examined 2 hours after shedding, the percentages were 50, 40, 35, 20 and 10 + per cent respectively. When 3 hours old, there were 37 and 15 per cent ellipsoid eggs. When examined 5 hours after shedding, the percentages were 36, 35, and 29 per cent respectively.

These percentages are not strictly comparable. Age is one variable factor. In Table V, for example, the percentage of ellipsoid eggs decreased between ½ and 5 hours from 62 to 26 per cent. A second variable factor is the method of determining ellipsoid eggs. In Series *B* the two extreme diameters were measured in each egg. In other series these diameters were measured in eggs which could not definitely be classified by inspection. In other series, classification was by inspection only. Ellipsoid eggs may also appear circular in optical plane. Finally females were chosen (except Series *B*) with the least number of ellipsoid eggs.

Table V

This table illustrates the large percentage of ellipsoid eggs at the initial age, the degree of ellipsoidality, and the increased percentage of globular eggs with age. Eggs from 1 female aged at 19 ± 0.3° C. Series *B*1.

Difference in diameters (μ)	Age (*hours*). ½	2½	5	10½	23	28	32	48	52	56	72
Globular Eggs (per cent)											
0– .3	38	60	64	90	98	100	100	100	100	96	96
Non-globular Eggs (per cent)											
.4– .6	16	8	2	4	0	0	0	0	0	0	0
.7– 1.3	10	14	6	2	0	0	0	0	0	0	0
1.4– 2.5	14	12	6	0	0	0	0	0	0	4	2
2.7– 3.7	6	4	10	2	0	0	0	0	0	0	0
3.8– 5.0	8	0	6	4	0	0	0	0	0	0	0
5.1– 7.5	2	0	2	0	0	0	0	0	0	0	0
7.6–10.0	2	0	2	0	0	0	0	0	0	0	0
10.1–12.5	4	0	0	0	1	0	0	0	0	0	0
12.6–15.0	0	2	2	0	0	0	0	0	0	0	2
No. Eggs Measured	74	50	50	51	50	50	50	50	50	40	40
Cleavage (*per cent*)	99	—	—	96	91	—	90	60	—	12	6

Hence it may be concluded that ellipsoid eggs occurred in all females studied; that the percentage ranged from 10 + to 62; that the actual percentages are probably greater. Since cleavage was normal and ranged from 97 to 100 per cent [3] (except Series G_1), neither ellipsoid shape *per se* nor the increased numbers with ellipsoid shapes are criteria of physiological deterioration.

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[3] The eggs of the other females were also in good physiological condition, for over 95 per cent formed parthenogenic membranes in distilled water.

Increasing Globular Shape with Age

Series *B* (Table V) illustrates the increasing globular shape with age. The eggs of one female were measured at different ages between ½ and 72 hours after shedding. The two extreme diameters were measured in consecutive eggs, 40 to 74 at each age. When ½ hour old, only 38 per cent were globular, i.e., the extreme diameters at right angles to each other differed by 0 to 0.35 μ. Of the 62 per cent ellipsoid eggs, 16 per cent had diameters differing by 0.4 to 0.6 μ, 10 per cent differed by 0.7 to 1.3 μ, 14 per cent by 1.4 to 2.5 μ, etc. The most ellipsoid eggs had diameters differing by 15.0 μ.

At successive ages between 2½ and 28 hours, the percentage of globular eggs increased progressively, viz., from 38 to 60, 64, 90, 98, and 100 per cent respectively. All eggs remained spherical between 28 and 52 hours.

Though only 38 per cent of the freshly-shed eggs were spherical, yet they were in good physiological condition; 99 per cent cleaved normally. During the next 10 hours, the percentage of spherical eggs increased from 38 to 90 per cent. During this time there was little or no deterioration. Cleavage was normal and decreased from 99 to 96 per cent, probably due to delayed rather than reduced cleavage.

Between 28 and 52 hours, there was no change in shape, yet deterioration was very rapid. Cleavage was increasingly irregular and fell from 91 to 34 per cent. Between 56 and 72 hours, with extreme deterioration 4 per cent ellipsoid eggs reappeared.

Seven other series gave essentially similar results.

Factors Involved in Change of Shape

Unripe eggs increase in size and are spherical before maturation. It is generally assumed that eggs are normally shed soon after maturation, but eggs may be retained within the body for long periods and be deteriorated when shed. This is indicated by the following: *Arbacia* were kept in separate large aquaria, the water changed twice daily. When shed the eggs were immediately inseminated. Such eggs frequently gave low percentage cleavage. Extreme deterioration was also observed in eggs from freshly-opened females, collected after severe storms or extreme heat, also towards the close of the breeding season.

It is believed that any condition that delays shedding of eggs results in increasing the number and size of ripe ova. As a consequence there is an increasing mechanical pressure of eggs against one another; and it is this pressure that gives rise to increasing ellipsoid shape. The greater the duration and degree of pressure, the greater the number of ellipsoid eggs and the greater the degree of ellipsoidality. This mechanical pressure exerts little or no injurious effect. Injury appears to be due to ageing processes within the body of the female. Upon shedding, the mechanical pressure is released, and increasing permeability to water further aids in the return to globular shape.

CONCLUSIONS

Cyclical Change in Size

The average size of freshly-shed eggs, in good physiological condition, varied widely in the 25 females studied, viz., 177,600 to 243,100 μ^3, or 36.9 per cent.

The eggs from any one female varied within much narrower limits, viz., 13,600 to 38,700 μ^3, or 12.6 per cent.

Size of eggs increased with age in all 13 series.

The increase may be evident by the third hour and end between 23 and 50 hours.

The increase ranged from 1.1 to 5.8 per cent. These percentages exclude fused and dead eggs.

The minimum and maximum, the mode and average, as well as the number of large eggs, all increased progressively with age.

During late ages, size decreased progressively by 4.2 to 9.7 per cent. This excluded fragmented eggs.

Size increase was accompanied by little deterioration; size decrease by considerable deterioration.

Increasing size is attributed to increased water intake; decreasing size to ejection of pellicles and water.

The cyclical change in size of control eggs must be known in the determination of the permeability rate.

Change in Shape

Ellipsoid eggs occurred in all 16 females. They ranged from 10 + to 62 per cent. The degree of ellipsoidality is given in text.

These females were selected for least ellipsoid eggs.

Ellipsoid eggs were in excellent physiological condition.

With age, eggs became spherical. All eggs were spherical in 8 to 37 hours, and remained spherical thereafter.

Shape is not correlated with physiological condition.

Ellipsoid shape is attributed to retention of mature eggs within the body, and therefore to increased numbers and size. The result is mechanical pressure and ellipsoidality. Shedding releases this pressure. Increased water intake aids in rounding out and enlargement of eggs.

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BIBLIOGRAPHY

GLASER, O. C., 1914*a*. *Biol. Bull.*, **26**: 84.
GLASER, O. C., 1914*b*. *Biol. Bull.*, **26**: 367.
GLASER, O. C., 1924. *Biol. Bull.*, **47**: 274.
GOLDFORB, A. J., 1913. *Biol. Bull.*, **24**: 73.
GOLDFORB, A. J., 1917*a*. *Carnegie Inst. Publ.*, No. 251, p. 73.
GOLDFORB, A. J., 1917*b*. *Proc. Nat. Acad. Sci.*, **3**: 241.
GOLDFORB, A. J., 1918*a*. *Biol. Bull.*, **34**: 372.
GOLDFORB, A. J., 1918*b*. *Biol. Bull.*, **35**: 1.
GOLDFORB, A. J., 1929*a*. *Biol. Bull.*, **57**: 333.
GOLDFORB, A. J., 1929*b*. *Biol. Bull.*, **57**: 350.
GOLDFORB, A. J., 1929*c*. *Biol. Bull.*, **57**: 389.
HARVEY, E. B., 1932. *Biol. Bull.*, **62**: 155.
HARVEY, E. N., 1932. *Biol. Bull.*, **62**: 141.
LILLIE, R. S., 1916. *Jour. Exper. Zool.*, **21**: 369.
LUCKÉ, B., AND M. MCCUTCHEON, 1926. *Jour. Gen. Physiol.*, **9**: 709.
LUCKÉ, B., H. K. HARTLINE, AND M. MCCUTCHEON, 1931. *Jour. Gen. Physiol.*, **14**: 405.
MCCUTCHEON, M., AND B. LUCKÉ, 1926. *Jour. Gen. Physiol.*, **9**: 697.
MCCUTCHEON, M., AND B. LUCKÉ, 1927. *Jour. Gen. Physiol.*, **10**: 659.

VISCOSITY CHANGES IN AGEING UNFERTILIZED EGGS OF ARBACIA PUNCTULATA

A. J. GOLDFORB

With Technical Assistance of Milton Landowne

(*From the College of the City of New York*)

In previous studies it was demonstrated that ageing unfertilized eggs of *Arbacia punctulata* undergo a series of changes including rate of fertilization, membrane formation, and cleavage (Goldforb, 1918*a* and *b*), agglutinin concentration (Goldforb, 1929*a* and *b*), and size (Goldforb, in press). Permeability to water and stretching and bursting of the plasma membrane in hypotonic sea water changed markedly with age. These studies will be reported later. The present study is devoted to a consideration of the viscosity changes.

Procedure

Eggs from each female, if in good physiological condition, were washed in 200 cc. sea water. Aliquot portions were transferred to flat finger bowls with 150 cc. sea water. Morning and evening the supernatant water was removed, the eggs transferred to clean bowls, and sea water added. Sea water was collected in quantity at high tide, filtered and stored. The pH was 8.3. The temperature in the bowls varied from 18 to 22° C., but during an experiment within 2.5°, usually within 1.5° C. At successive ages eggs from the same bowl were centrifuged by a slight modification of the method developed by Heilbrunn (1928).

Constant Centrifugal Force, Varying Time, Early Experiments

In 1930,[1] eggs were centrifuged in 2 mm.-bore tubes in a small electric centrifuge. Immediately after centrifuging, the eggs were examined in sea water. After preliminary trials a period of centrifugation was chosen that gave the following zonation:

(1) Oil cap sharply defined.

(2) Hyaline zone in most eggs extended 45 to 55° [2] from center of oil cap.

(3) Twenty consecutive eggs, with planes of zoning at right angles to the horizontal, were measured.

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[1] Dr. V. Schechter assisted in these experiments.

[2] I.e., degrees on circumference from center of oil cap.

(4) Hyaline zone near oil cap free of granules, near grey zone containing numerous scattered granules.

(5) Grey and red zones sharply differentiated.

At successive ages, eggs were centrifuged at the same speed, but the time was varied to approximate the same degree of zonation. Increase in time denoted relative increase in viscosity, and vice versa. The results may be summarized as follows:

Between 10 minutes and 3 hours after shedding, viscosity increased in most experiments. The exceptions will be discussed later.

Between ca. 3 and 30 hours, *viscosity progressively increased with age* in every experiment. For example, in one experiment the time of centrifuging required to approximate the same degree of zonation increased progressively from ¾ to 2½ minutes.

Beginning about 40 hours after shedding, *viscosity progressively decreased.*

This cyclical change in viscosity occurred in all experiments. That the changes were significant was shown by the following: Repetitive tests agreed within 15 seconds; centrifuging time increased with age by 105 or more seconds. Though the temperature during some tests rose to 3½° C., yet when those tests were chosen in which the temperature did not vary beyond 0.5°, the same changes occurred. Though the number of eggs examined at each age was small, yet all experiments gave similar results.

Constant Force and Time, Change in Percentage of Zoned Eggs

In 1931, with the same centrifuge and tubes, the centrifugal force and time were constant at successive ages. The centrifuge registered 4,444 r.p.m. (without load). As the result of preliminary trials a period of centrifugation of 2 minutes duration was usually chosen. This time included 7½ ± ½ seconds to attain maximal speed, but did not include 26 ± 2 seconds for the centrifuge to stop. The voltage was constant during the day. Tests were made when no other large electrical apparatus was used on the same line. During a test the temperature in the centrifuge head varied between 21.4° and 22.4° C., usually within 0.6°. Immediately after centrifuging, the eggs were transferred to 0.4 per cent formalin (Howard, 1931) and examined within 2 hours. About 100 consecutive eggs, lying at a proper angle, were examined at each age and classified as follows:

Group 1	*Group 2*	*Group 3*
Oil cap very distinct	Same	Less distinct
Hyaline zone, granules few	Many	Very numerous
Hyaline zone, 60–70°	45–60°	Less than 40°
Hyaline grey border, thin straight line	Very ragged	Loose band
Hyaline and grey zones sharply different in color	Less sharp	Intergrades
Oil to grey zone, ca. 15 units	Ca. 12	Less than 10

A typical experiment is summarized in Fig. 1. The eggs of one female were centrifuged for one minute. The number of eggs recorded at each age varied from 84 to 247 (average 194). Between 2¾ and 22 hours after shedding, the adequately zoned (Group 1) eggs *decreased* progressively from 51 to 14 per cent. This decrease denoted correspondingly greater viscosity. After 22 hours, however, there was a reversal. The percentage of Group 1 eggs *increased* from 14 to 51 and then to 84 per cent. This reversal or liquefaction occurred during rapid deterioration.

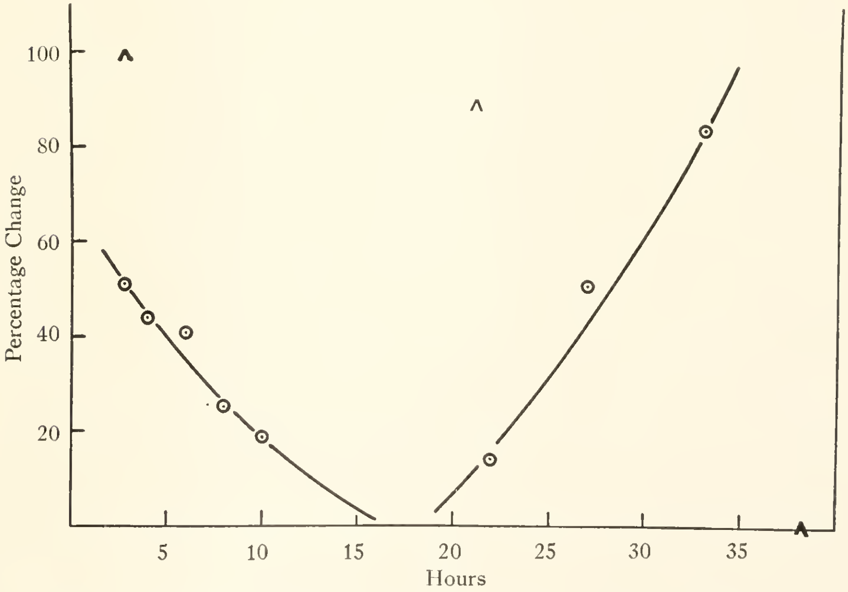

FIG. 1. Centrifuged at 4,444 r.p.m. for 1 minute at 21.5° C. The percentage of eggs zoned to a fixed standard decreased progressively during the first ca. 20 hours and increased thereafter. A decrease in percentage indicates corresponding increase in viscosity and vice versa. ⊙ = per cent zoned; ∧ = per cent cleavage of control eggs.

Similar results were obtained in a second experiment. Eggs 1½ hours old, centrifuged for 2 minutes, gave 85 per cent which were sharply zoned. Between 1½ and 29 hours the percentage of eggs of Group 1 decreased progressively from 85 to 0 per cent, while the percentage of eggs of Group 2 increased from 5 to 94 per cent. When 48 hours old, Group 2 eggs had decreased from 98 to 38 per cent, while Group 3 (the least zoned) eggs had increased from 6 to 62 per cent. In the case of the older eggs, in order to avoid crushing them at the bottom of the tubes, second samples were centrifuged for one minute only. Between 24 and 48 hours the eggs of Group 2 decreased progressively from 91 to 24 per cent, while Group 3 eggs increased from 9 to 76 per cent.

In a third experiment, the percentage of adequately zoned (Group 1) eggs decreased, between 5 and 48 hours, from 100 to 71 per cent. When centrifuged during late ages, for 1 minute only, the percentage of Group 2 eggs decreased from 85 to 17 per cent.

In the last two experiments liquefaction appears to have begun about the forty-eighth hour. At this age the zonation of many eggs was greater and the hyaline zone contained fewer granules and was much deeper than at any previous age.

It was therefore concluded that viscosity was progressively increased during early and intermediate ages; that there were indications of a progressive liquefaction during late ages; that the degree of change is indicated by the change in percentage of eggs zoned to a fixed standard.

Same Force, Varying Time, Improved Technique

In 1932, viscosity was determined by the following improved technique:

1. *Temperature.*—The centrifuge head was cooled by a regulated flow of ice water. The temperature was recorded at the beginning and end of each test. It varied within 1°, usually within 0.5° C.

2. *Centrifugal Force.*—A powerful Emerson hand centrifuge was used. The time necessary to attain maximum speed was reduced from 7½, in preceding experiments, to 1 second. The time necessary for stopping was reduced from 26 to 1 second.

3. *Low Centrifugal Force.*—After repeated trials a relatively low centrifugal force was selected, viz., 1,750 gravities.

4. The standard of zonation approximated that of Group 2 in previous experiments. At this low centrifugal force and short duration, a small change in centrifuging time gave rise to readily detectable changes in zonation. Hence accuracy of tests was increased.

5. Two or more determinations were made at each age, one usually for the same time as in the accepted previous test, the other for longer

or shorter time. *The objective was to produce not only the same zonation, but the same percentage of similarly zoned eggs.*

6. *The Avoidance of Injury at Bottom of Tube.*—To prevent smashing of eggs at the bottom of the tube, at late ages, a capillary drop of eggs was added to a fixed level of isosmotic solution of C.P. cane sugar solution. The eggs were not injured by the sugar solution, for they cleaved like the control eggs. Nor was their viscosity altered by the solution.

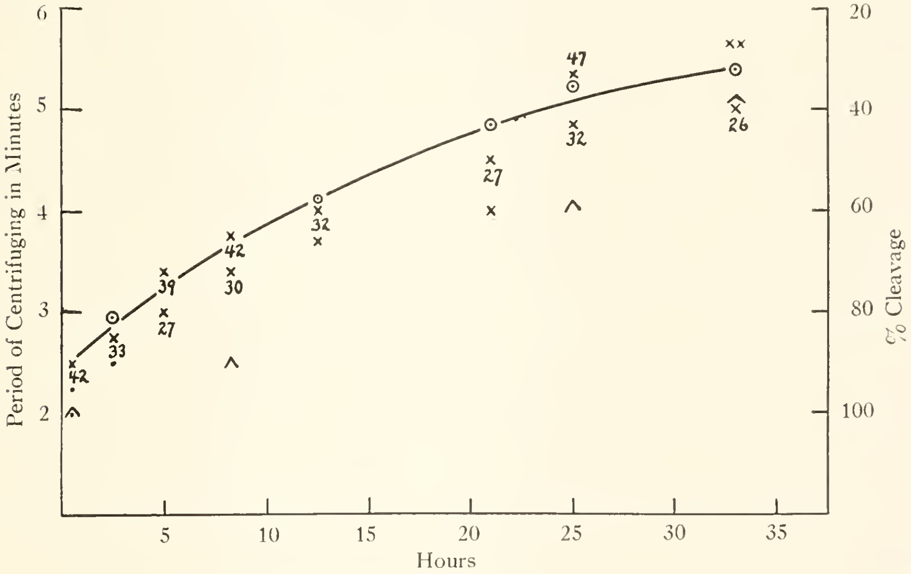

FIG. 2. Centrifuged at 1,750 gravities at 21.5° C. for indicated minutes to approximate same percentage of similarly zoned eggs. Centrifuge time increased during the first 33 hours.

× = observed time in minutes.
⊙ = calculated time.
· = insufficiently zoned.
×× = overzoned.
∧ = percentage cleavage.
Numbers = percentage of eggs zoned to standard.

7. *Sensitivity of Test.* An increase of 10 to 15 seconds longer centrifuging definitely increased the percentage of zoned eggs. For example, in one experiment, eggs centrifuged for 2 minutes resulted in no eggs zoned to standard. With each increase of 10 seconds centrifugation, the percentage of zoned eggs increased to 18, 53, and 86 per cent respectively. In another experiment centrifugation for 1¾ min-

utes produced 34 per cent zoned eggs. Fifteen seconds more centrifugation gave 52 per cent. Repetitive tests varied 0 to 8 per cent.

Cyclical Viscosity, Eggs Centrifuged in Sea Water

In Experiment T_1 (Fig. 2), the first test was made 17 minutes after shedding. When centrifuged for 2 and 2¼ minutes, the eggs were insufficiently zoned. When centrifuged for 2½ minutes, 42 per cent (of 165 eggs) were zoned to a carefully noted standard.

When 2½ hours old, one sample was tested for 2½ minutes as before. The eggs were not sufficiently zoned. The second sample, centrifuged 30 seconds longer, contained only 33 per cent zoned to standard. The estimated time necessary to produce the same percentage of eggs zoned to the same standard as in the initial test was 2′ 55″.

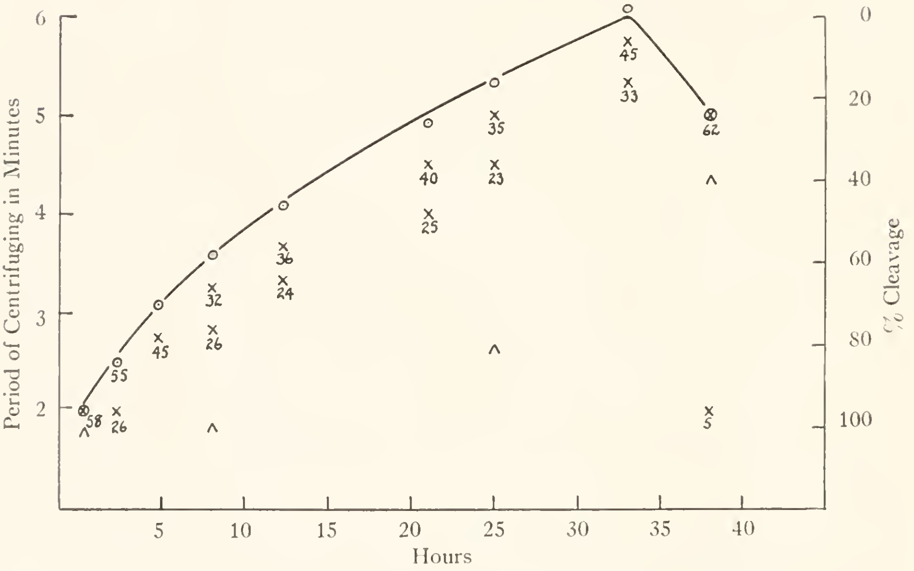

Fig. 3. Same conditions and symbols as in Fig. 2. Shows *increased* viscosity during first 33 hours and decreased viscosity thereafter.

When 5 hours old, 2 samples were again centrifuged, one for 3 minutes (to approximate the previous estimated 2′ 55″) and another for 3′ 25″. The first contained only 27 per cent, the second 39 per cent zoned eggs. The latter approximates the initial 42 per cent. Hence 55 seconds longer centrifuging was required at this age to produce the same zonation as in the ¼-hour old eggs.

Similar double tests were made at successive ages to 33 hours. The time required to approximate the same percentage of equally zoned eggs increased with age as follows: 2.5, 2.7, 3.4, 3.7, 4.0, 4.5, 5.3, and 5.5 minutes respectively. The estimated time increased progressively from 2½ to 5⁵⁄₁₂ minutes (Fig. 2). Viscosity increased 116 per cent.

The experiment was repeated with similar results (Fig. 3). Several facts deserve brief mention.

(1) For similar ages the increase in centrifugal time was greater, i.e., from 2 to 6¹⁄₁₂ minutes, an increase of 202 per cent.

(2) Liquefaction occurred after 33 hours. Between 33 and 38 hours the centrifugal time decreased from 6½ to 5 minutes. Though the eggs were less viscous than at the thirty-third hour, they were much more viscous than at the initial age, for when centrifuged for 2 minutes (the initial time) only 5 instead of 58 per cent of the eggs zoned to standard.

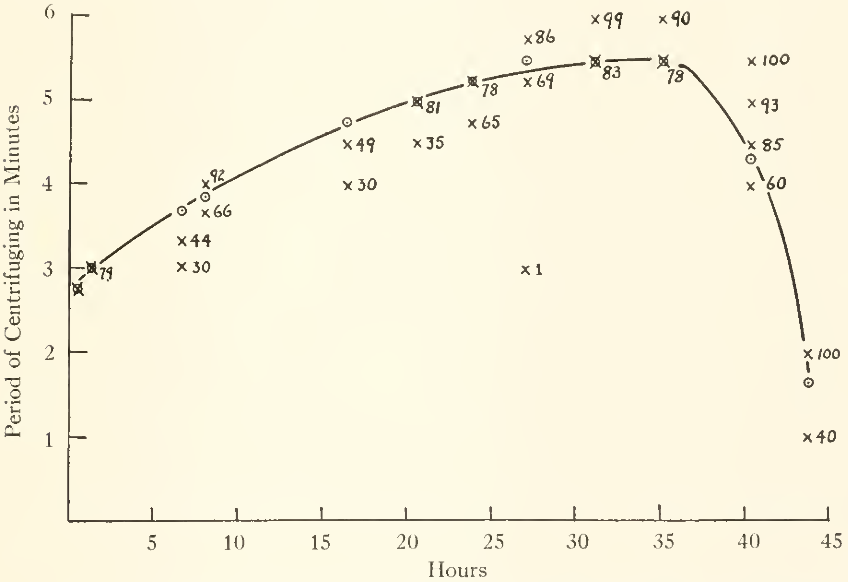

Fig. 4. Centrifuged in isosmotic sugar solution 1,460 gravities. Same symbols as in Figs. 2 and 3. Shows increased viscosity to thirty-fifth hour and progressive decrease to forty-fourth hour.

(3) Liquefaction occurred when the eggs were markedly deteriorated.

Cyclical Viscosity, Eggs Centrifuged in Isosmotic Solution

When centrifuged in an isosmotic C.P. cane sugar solution (density 1.085), the eggs were scattered below the sugar level, where the force was 1,460 ± 100 gravities.

In Experiment T_3, for example, 2 to 6 tests were made at each age. An average of 173 eggs were examined after each test. Between 1½ and 35 hours the time of centrifuging, required to produce the same percentage of equally zoned eggs, increased progressively from 2¾ to 5½ minutes (Fig. 4).

When eggs were 27 hours old, it was necessary to centrifuge for 5½ minutes to zone 79 per cent (the initial per cent) to standard. When centrifuged for 3 minutes (the initial duration), only 1 per cent were zoned to standard. This affords another measure of the increased viscosity at this age.

After 35 hours there was rapid liquefaction. When 40½ hours old, a sample centrifuged for 5½ minutes gave 100 per cent either zoned to standard or overzoned. Less centrifugation, i.e., 4⅓ minutes, sufficed. When 43½ hours old, eggs were centrifuged for 4½, 4, 3½, 3, and 2 minutes respectively. All these gave 100 per cent zoned or overzoned eggs. The time required to zone to standard had decreased from 5½ to 1⅔ minutes.

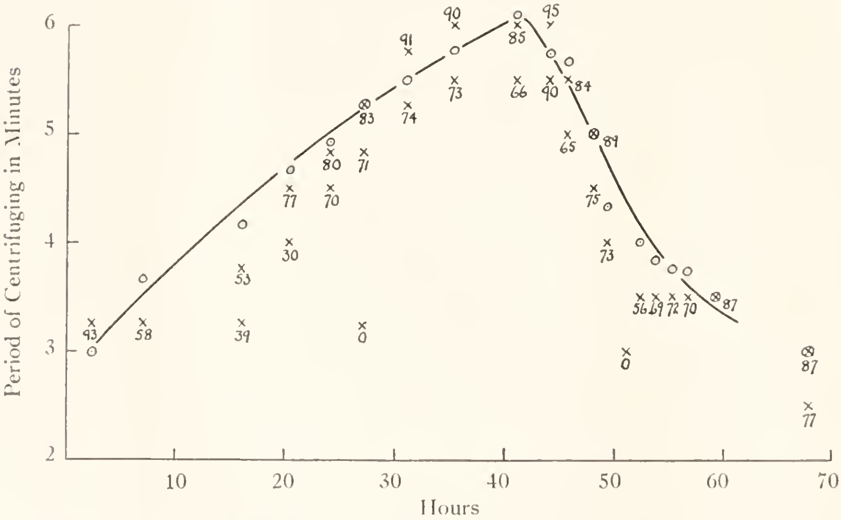

FIG. 5. Another experiment as in Fig. 4, but for more ages. Same cyclical viscosity with age.

In Experiment T_4, Fig. 5, tests were made as in the previous experiment but over a longer series of ages. Viscosity increased progressively during the first 40 hours. The increase was from 3 to 6$\frac{1}{12}$ minutes or 100 per cent. Between 44 and 68 hours viscosity decreased rapidly. At late ages, even at the reduced time of centrifugation, nearly all the eggs were overzoned, so that liquefaction was greater than indicated by the figures.

It is therefore concluded that *ageing eggs undergo a cyclical change in viscosity, increasing during the first ca. 35 hours and decreasing thereafter,* and that the increase is of the order of 2 to 3 times the initial viscosity. The subsequent decrease is even greater.

Viscosity of Ripe vs. Unripe Eggs

In the preceding experiments there were a few unripe eggs in nearly every test. None of these, however, showed any trace of zonation. To determine the relative viscosity of ripe and of unripe eggs, females were chosen that contained the largest number of unripe eggs, and were subjected to longer durations and greater centrifugal forces. Table I summarizes 3 experiments.

Table I

Viscosity of Unripe Eggs

Exp. No.	♀ No.	Age	Centrifugation		Medium	Zoning Unripe Eggs	Zoning Ripe Eggs
			Force	Time			
		hours	*gravities*	*minutes*			
1	1	2	1750	1⅚	Sea Water	0	68% zoned to standard
	2	4	1750	2	" "	0	67 " " "
2	1	1	710	7½	Isosmotic Sugar Sol.	0	30 " " 90°
	1	2	710	10	" " "	0	50 " " "
	1	3	710	15	" " "	0	100 " " 110°
	1	4	710	25	" " "	0	100 " " 120°
3	1	2⅙	1750	1¾	" " "	0	Most " " 45°
	1	1⅚	1750	2	" " "	0	" " " 55°
	1	1¾	4000	1½	" " "	0	" " " 65°
	1	1⅔	4000	2½	" " "	0	" " " 75°
	1	1½	7000	3	" " "	0	" " " 90°
	1	1⅙	7000	5	" " "	0	" " " 110°

Experiment I

Eggs were centrifuged in sea water at 1,750 gravities for 2 minutes. Sixty-eight and 67 per cent of 400 ripe eggs were zoned to standard. None of the unripe eggs in a sample of 1,600 eggs showed any evidence of zonation.

Experiment II

Eggs were centrifuged at 710 gravities for 7½ to 25 minutes. When centrifuged for 25 minutes, all ripe eggs were much overzoned; the hyaline grey border extended from 90 to 120°; yet none of the unripe eggs showed any trace of zonation. A few small unripe eggs formed pseudopodial processes.

Experiment III

Eggs were centrifuged at increasing forces, viz., 1,750, 4,000 and 7,000 gravities for 1¾ to 5 minutes. With each increase in time or force the hyaline zone deepened and the number of scattered granules therein decreased. At the greatest centrifugal force all ripe eggs were not only overzoned but elongated considerably, as described in detail by E. B. Harvey (1932). *Not a single unripe egg was elongated, and none showed any sign of zonation.*

It is therefore concluded that *either the gravimetrically separable substances are not organized in the unripe egg or being present the viscosity is more than eleven times greater than in ripe eggs.*

Unripe eggs vary in size to a very much greater degree than ripe eggs, yet no difference in viscosity was detectable between the very small (young), and the large (older) unripe eggs. These observations suggest that, with maturation, there is not only a very profound but possibly a very rapid liquefaction. Liquefaction appears to be far greater than during polar body formation or cleavage, or following changes of temperature, salts, etc. (Heilbrunn, 1921, 1924, 1928).

Discussion

It was shown above that in the life history of the *Arbacia* egg there were five viscosity phases, viz.:

1. *Extreme Viscosity, Unripe Eggs.*—Whatever their age (or size) all unripe eggs were extremely viscous. None could be zoned by the considerable forces and durations used. That unripe eggs were more viscous than ripe ones was noted by Paspaleff (1927) and by Heilbrunn (1928). Our study corroborates these findings and supplies a quantitative measure of the difference, namely that unripe eggs are at least 11 times more viscous than freshly-shed ripe eggs.

2. *Liquefaction.*—Upon maturation there was a very marked and probably very rapid liquefaction in all eggs.

3. *Increasing Viscosity, Ripe eggs, Early Ages.*—All ripe eggs progressively increased in viscosity with age. The rate of increase was slower when the eggs were aged within the body, faster when they were aged in sea water. Viscosity increased during the first ca. 35 (22 to 41) hours after shedding. The increase was 2 to 3-fold.

4. *Decreasing Viscosity, Ripe Eggs, Late Ages.*—After ca. 35 hours there was a reversal, namely, a progressive liquefaction. The rate was faster and the total change was greater than during earlier ages.

5. *Extreme Viscosity, Death.* Our evidence is not conclusive whether at the greatest ages death was directly accompanied by coagula-

tion or whether extreme liquefaction occurred first, followed quickly by coagulation.

These five phases are diagrammatically represented in Fig. 6.

Extent of Change.—These viscosity changes are greater than those which occur under a variety of other conditions, but comparisons are not readily made, either because the changes have not been given quantitatively or because different units have been used. In Table II such a comparison is attempted. This table shows that viscosity increased during mitosis of *Arbacia* eggs by two-thirds. Larger changes occurred with temperature, HCl, NH_4OH, ultraviolet light, etc., viz., 2¼ to 4 x. The largest change heretofore described occurred during mitosis of *Cumingia eggs,* viz., 5 to 8x. During ageing of *Arbacia* eggs, there were both positive and negative changes of 2 to 11x.

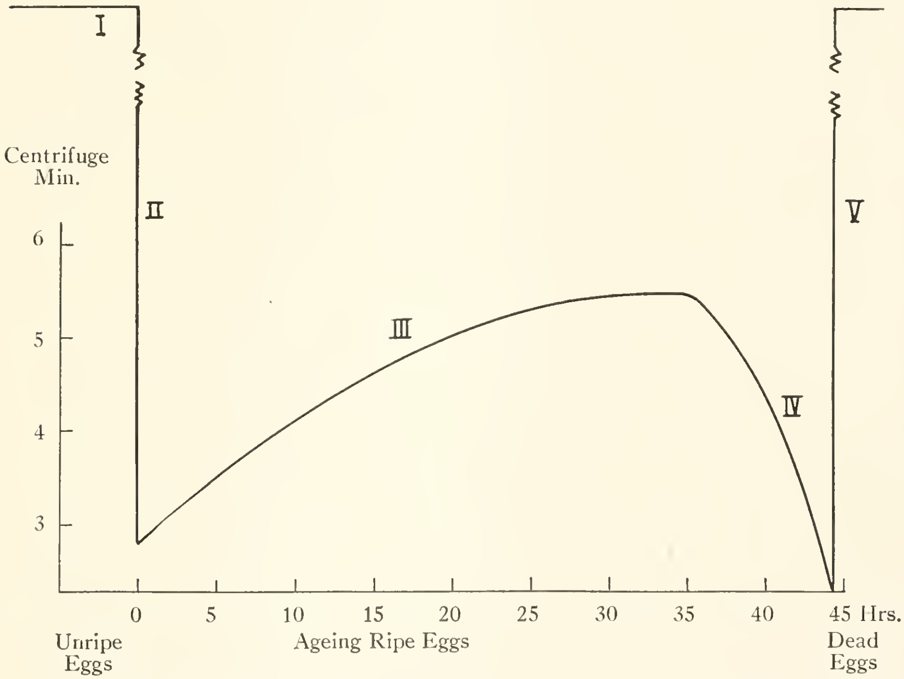

Fig. 6. Diagrammatic representation of the five phases in viscosity of the egg, viz.

I. All unripe eggs at any age, extremely viscous.
II. Maturation, extreme and rapid liquefaction.
III. Ripe eggs, early and intermediate ages, progressively increased viscosity.
IV. Ripe eggs, late ages, much deterioration, progressive liquefaction.
V. Dead eggs, coagulation.

The factors which play a minimal or no rôle in these changes include body fluid, temperature, and pH.

Modification of Egg by Body Fluid.—Body fluid decreases cleavage (Lillie, F. R., 1923; Ephrussi, 1925). Boguchi (1930) believed that this was due to intestinal, not to body fluid. Body fluid alters the reaction of eggs to calcium (Heilbrunn, 1925, 1928). In a later study it will be shown that body fluid does decrease cleavage, gives rise to irregular cleavage, accelerates swelling in hypotonic solutions, and increases viscosity. In these experiments body (perivisceral) fluid was withdrawn by pipette through the oral disc, centrifuged, and the supernatant fluid used. The freshly-shed "dry" eggs or eggs from removed ovaries were immediately transferred to this fluid. At successive ages one sample of eggs washed in sea water and another unwashed sample were centrifuged simultaneously in sea water. The body fluid eggs were in every experiment more viscous than eggs from which the body fluid had been washed away. The difference was small but definite. By ca. the sixth hour, viscosity was the same in both kinds of eggs. Therefore neither the marked liquefaction upon maturation, nor the striking increase in viscosity with age may be attributed to the effect of body fluid. It was pointed out above that in a few of the early experiments there was no rise in viscosity during the first 5 hours. This we believe was due to a comparison of eggs whose body fluid had not been thoroughly washed away (hence with greater viscosity) with eggs at the next age when washing had been complete.

TABLE II

Comparison of Viscosity Changes under Various Conditions

Author	Inducing Conditions	Cell	Viscosity Change
Heilbrunn, 1920	Mitosis	Arbacia Eggs	+ 2/3
Pantin, 1924	Temperature −0.7 to 30° C.	Nereis "	− 3 2/5
Heilbrunn, 1921	" 2 to 15° C.	Cumingia "	+ 2 1/4
" "	" 15 to 30° C.	" "	− 2 1/4
Barth, 1929	CO_2, pH 8.3 to 5.0	Arbacia "	+ 2 1/2
	HCl " 8.3 to 3.0	" "	+ 2 1/2
	NH_4OH " 8.1 to 11.0	" "	+ 4
Heilbrunn and Young, 1930	Ultra-violet ray	" "	+ 3 to 4
Heilbrunn, 1921	Mitosis	Cumingia "	+ 5 to 8
Goldforb	Unripe eggs	Arbacia "	+11
	Ripe eggs, early ages	" "	+ 2 to 3
	" " , late "	" "	− 3 to 4
	Dead "	" "	+11

Temperature and Viscosity.—In *Nereis* eggs a rise in temperature from — 0.7 to + 30° C. decreased viscosity 3½ times (Pantin, 1924). In *Cumingia* eggs a similar increase in temperature gave rise to a cyclical change in viscosity with maxima at 0, 15, and 32° C. (Heilbrunn, 1924). In our experiments there were five phases with maximal changes of 11 times, but the variation in temperature was within 2.25° C. When those tests were selected during which the temperature had not changed more than 1 or 0.5° C., the same viscosity changes occurred. Temperature, therefore, did not give rise to the marked viscosity changes in ageing eggs.

Hydrogen Ion Concentration.—Acid tends to increase, alkali to decrease viscosity (Jacobs, 1922; Barth, 1929); but viscosity begins to change in acid medium below pH 7.8 (Barth, 1929). In our experiments the transfer from body fluid to sea water decreased viscosity slightly. This was probably due to the change from the acid body fluid, viz., pH 7.4, to alkali sea water, viz., pH 8.3. With ageing, the pH of the supernatent sea water decreased slowly but not below 7.9. Hence this change in hydrogen ion concentration *per se* is not a significant factor in the large viscosity changes during ageing. During latest ages, liquefaction may possibly be due in part to the rapid and considerable inflow of sea water rendering the interior less acid.

Granule Size.—Increased viscosity may be due to the formation of new granules or the increased size of old granules (Heilbrunn, 1928). We were unable, as were previous workers, to demonstrate such changes. Conversely the decreased viscosity during latest ages may be due to diminution of the granules in one or more layers. The four zones[3] were present at all ages, but their volumes changed during latest ages. The oil zone appeared to be unchanged. The hyaline zone, however, was much larger, in extreme instances two or more times the size at previous ages, when the same forces and durations were used. This zone was entirely free of grey granules. More significant is the diminution of both grey and red zones. They shrank from a previous ca. 120°[4] to about ⅓ or 45° in depth. This may mean either a diminution in size of the same number of granules or a loss in number of granules or both. Either supposition may account for the liquefaction at these late ages, but due to this change in depth of zones, an exact comparison of the viscosity at these late ages with the viscosity at earlier ages may not therefore be made.

Ion Penetration.—Rate of ion penetration may change with age. Osterhout (1926) and Brooks (1929) found that K accumulated in the cell sap of ageing *Valonia*. Stiles and Kidd (1919), studying the relative penetration of K, Ca, Na, and Mg in ageing carrot and potato cells, discovered that calcium changed most with age. The effect of changing concentration of ions with age requires further study. The calcium change is now under investigation.

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[3] The fifth zone, described by E. B. Harvey (1932), was not studied.

[4] Measured in degrees on the periphery.

Permeability.—Viscosity and water intake both increased during mitosis and cell division (Heilbrunn, 1920*a*; Haberlandt, 1919, 1920). In our next study it will be shown that permeability to water progressively and markedly increased in ageing *Arbacia* eggs. Increased viscosity in these eggs occurred with increasing permeability to water. Both increased during the first ca. 35 hours after shedding. Thereafter permeability increased much further, while viscosity was reversed. Similar reversal of viscosity occurred with increasing CO_2 (Jacobs, 1922), increasing alkalinity (Barth, 1929), increasing temperature (Heilbrunn, 1920*b*, 1924). It is extremely interesting that Dhar (1930) obtained a reversal of viscosity in ageing hydrophyllic colloids. In our experiments the liquefaction at late ages may be due in part to excess water, to decreased acidity of interior of egg, or to excess calcium.

Injury.—Without attempting to define injury, it may be said that increasing permeability and viscosity occurred with little or no protoplasmic deterioration. Deterioration was measured by decreased and by irregular cleavage. In Experiment T_2, Fig. 3, for example, between 1/4 and 8 hours, the viscosity increased 79 per cent, i.e., the centrifugal time necessary to zone to standard increased from 120 to 215 seconds. During these ages cleavage was normal and decreased but 1 per cent (i.e., from 100 to 99). Between 8 and 33 hours viscosity increased to 204 per cent. Irregular cleavage began at the twenty-first hour. Cleavage decreased at the thirty-third hour to 80 per cent. Viscosity, during these ages, increased with progressive injury. Buenning (1926) had observed this phase only. During later ages there was considerable and rapid deterioration. This was associated with decreased viscosity.

SUMMARY

1. Relative viscosity was determined by (*a*) change in percentage of eggs zoned to a fixed standard, at constant centrifugal force and time; (*b*) change in time at a given force to produce same percentage of equally zoned eggs.

2. Ageing unfertilized eggs manifested five major phases in viscosity, viz.

(*a*) Unripe eggs. No trace of zoning could be induced. They were at least eleven times more viscous than mature eggs.

(*b*) All mature eggs were readily zoned at much lower forces and duration. Maturation is accompanied by profound and apparently rapid liquefaction.

(*c*) During the first ca. 35 hours after shedding, ripe eggs progressively *increased* in viscosity, 2 to 3 ×. At constant force and time, the zoned eggs decreased with age from 85 to 0 per cent. To produce the same percentage of equally zoned eggs, the centrifuging time increased from ca. 2 to 5 minutes. Similar results were obtained when eggs were centrifuged in isosmotic sugar solution.

(*d*) With further ageing, viscosity was reversed. There was progressive liquefaction. The decrease was 3 to 4 ×.

(*e*) Upon death viscosity was again sharply increased at least eleven times.

3. The amount of change with age is greater than that during mitosis, or induced by heat, acid, light, etc.

4. Temperature and acid are excluded as factors in the cyclical viscosity changes with age.

5. Perivisceral fluid is also not responsible.

6. Increasing permeability to water is associated with increasing viscosity during early and intermediate ages. During late ages, with excess water intake, viscosity is reversed.

7. Viscosity increased during early ages with no detectable injury. It increased further with progressive injury, i.e., during intermediate ages. It decreased during the period of most rapid injury.

BIBLIOGRAPHY

Barth, L. G., 1929. *Protoplasma,* **7**: 505.
Boguchi, M., 1930. *Protoplasma,* **11**: 432.
Buenning, E., 1926. *Bot. Arch.,* **14**: 138.
Brooks, S. C., 1929. *Protoplasma,* **8**: 389.
Dhar, N. R., 1930. *Jour. Phys. Chem.,* **34**: 549.
Ephrussi, B., 1925. *C. R. Acad. Sci.,* **180**: 775.
Goldforb, A. J., 1918*a*. *Biol. Bull.,* **34**: 372.
Goldforb, A. J., 1918*b*. *Biol. Bull.,* **35**: 1.
Goldforb, A. J., 1929*a*. *Biol. Bull.,* **57**: 333.
Goldforb, A. J., 1929*b*. *Biol. Bull.,* **57**: 350.
Goldforb, A. J., in press.
Haberlandt, G., 1919. *Sitzungsber. d. k. Preus. Akad. d. Wiss.,* Berlin, pp. 322 and 721; 1920, p. 323.
Harvey, E. B., 1932. *Biol. Bull.,* **62**: 155.
Heilbrunn, L. V., 1920*a*. *Jour. Exper. Zoöl.,* **30**: 211.
Heilbrunn, L. V., 1920*b*. *Biol. Bull.,* **39**: 307.
Heilbrunn, L. V., 1921. *Jour. Exper. Zoöl.,* **34**: 417.
Heilbrunn, L. V., 1924. *Am. Jour. Physiol.,* **68**: 645.
Heilbrunn, L. V., 1925. *Science,* **61**: 236.
Heilbrunn, L. V., 1927. *Arch. Exp. Zellf.,* **4**: 246.
Heilbrunn, L. V., 1928. The Colloid Chemistry of Protoplasm, Gebruder Borntraeger, Berlin.

Heilbrunn, L. V., and R. A. Young, 1930. *Physiol. Zool.,* **3**: 330.
Howard, E., 1931. *Biol. Bull.,* **60**: 132.
Jacobs, M. H., 1922. *Biol. Bull.,* **42**: 14.
Lillie, F. R., 1923. Problems of Fertilization, Chicago, p. 172.
Lillie, R. S., 1909. *Am. Jour. Physiol.,* **24**: 14.
Lillie, R. S., 1911. *Am. Jour. Physiol.,* **28**: 197.
Lillie, R. S., 1912. *Am. Jour. Physiol.,* **29**: 372.
Lillie, R. S., 1913. *Am. Jour. Physiol.,* **31**: 255.
Osterhout, W. J. V., 1926. *Proc. Soc. Exper. Biol. and Med.,* **24**: 234.
Pantin, C. F. A., 1924. *Jour. Marine Biol. Ass.,* **13**: 331.
Paspaleff, G., 1927. *Publ. d. Stazione Zool. d. Napoli,* **8**: 1.
Stiles, W., and F. Kidd, 1919. *Proc. Royal Soc.,* B., **90**: 487.

THE PHYSIOLOGY OF DIGESTION OF PLANKTON CRUSTACEA

I. Some Digestive Enzymes of Daphnia

ARTHUR D. HASLER

(*From the Limnological Laboratory, University of Wisconsin*)

This paper constitutes the first report of a biochemical assay of the digestive enzymes of *Daphnia*.

The food of plankton Crustacea has been a contestable subject since Pütter (1909) postulated that most aquatic organisms derived much of their nutrition from dissolved organic matter. The experiments contrary to this theory culminated in the work of Krogh (1930), who found dissolved organic substances of no importance in the nutrition of aquatic animals and of Stuart, et al (1931), who raised bacteriologically sterile *Moina* and found them unable to subsist on dissolved nutritives —particulate food alone could furnish a supporting diet. "Staubfeine" detritus appeared to Naumann (1918) to play the most important rôle in the nutrition of cladocerans. He also (1921) determined a renewal coefficient of the intestinal contents (15–30 minutes) in several species of the same group which demonstrated the short period that food remains in their digestive tracts. Klugh (1927) found some Entomostraca able to utilize fine detritus but showed their chief food to consist of planktonic Chlorophyceae. Many workers point to the importance of bacteria as food. It is not unreasonable to suppose that they make up a part of the filterable food of plankton Crustacea, for Juday (1934) calculates them to be 1 per cent of the dry organic matter found in the centrifuge plankton of Trout Lake.

Birge and Juday (1922 and 1934), in a chemical study of particulate and dissolved organic matter in southeastern and 529 northeastern Wisconsin lakes, find considerable available food stuffs. In the latter group of lakes the organic matter in the centrifugable plankton consisted of 37 per cent crude protein, 4 per cent ether extract and 59 per cent carbohydrate. In the former report, analyses of algae show them to have a higher protein content than carbohydrate.

The experiments reported in this paper show that a digestive mechanism exists in *Daphnia* capable of utilizing protein, carbohydrate, and fat.

Acknowledgment is appreciatively given to Professor C. Juday for suggesting the problem, to Professor H. C. Bradley, Department of Physiological Chemistry, under whose direction this work was carried out and who furnished laboratory facilities; also to Dr. H. D. Baernstein for instruction in the use of physico-chemical apparatus.

Materials and Methods

Daphnia magna was cultured in large battery jars and butter tubs, on a medium of sheep manure and acid phosphate. Frequent seining yielded sufficient *Daphnia* for limited analysis only. Pure cultures of *D. pulex* were netted from Lake Monona in the spring and fall of the year. Large quantities were obtained from this source.

Finely ground casein, on which the indicators neutral red and bromcresol-phenol had been adsorbed, was fed to *D. magna*. The color change of the indicator was observed in progress through the alimentary tract and the pH approximated. The pH was found to vary from 6.8 in the anterior end of the tract to 7.2 at the caudal end. This result indicated a tryptic type of proteolytic digestion. Rankin (1929) found a range of 6.8–8.0 in *Simocephalus*.

In the preparation of the extract, *D. pulex* were strained from the water, dehydrated and partially defatted by treatment with acetone. Defatting was continued with petroleum ether. One gram of residue was powdered in an agate mortar and extracted for twenty-four hours with 100 cc. of 50 per cent glycerol. The filtered extract contained proteinases, carbohydrate and fat-digesting enzymes. Fresh hog pancreas with a strip of duodenum were treated in the same manner as the *Daphnia*. This extract was compared with *Daphnia* extract in some of the experiments. For micro-analysis 50 intestines were dissected from *D. magna* and extracted with 50 per cent glycerol.

Examination of the Extract

The Proteinases

In order to micro-analytically detect the presence of a proteinase in *D. magna*, the Gates (1927) photographic plate method was modified to show qualitatively the proteolytic activity of the glycerol extract of *D. magna* intestines. Drops of this extract definitely digested the gelatin film of a photographic plate showing the presence of a proteinase in the digestive tract of *Daphnia*.

For quantitative analysis of the *Daphnia* proteinase, the viscosity method of Northrop (1922) was modified for use. With the aid of the Ostwald viscosimeter, it was possible to follow the hydrolysis of gelatin by a proteinase. The activity of the enzyme is measured in terms of decreasing viscosity (△V) of the substrate. A special gelatin from Swift and Co. was used for the first experiments. Solutions of 3 per cent concentration were made up in a phosphate buffer at pH 7.4, preserved in thymol and kept in test tubes (5 cc./tube) at 3° C. Test tubes of gelatin were placed in a water bath of 34° C.; the temperature was kept constant by a heating unit, agitator, and toluene regulator. When the gelatin reached bath temperature, it was transferred to Ostwald viscosimeters. To these were added 0.5 cc. of the 1 per cent glycerol extract. Viscosity readings were taken as often as possible for the first 15 minutes. After that, readings were made at 10-minute intervals for one hour. To the control tubes was added 0.5 cc. of 50 per cent glycerol. The gelatin-digesting ability of 1 per cent glycerol extracts of both *D. pulex* and hog pancreas were determined and compared. Fig. 1 shows the hydrolysis of gelatin by the proteinases in the extracts of both *Daphnia* and hog pancreas. The curves represent the mean of two runs against a control and were duplicated twice. They are typical hydrolysis curves, falling suddenly at the onset of cleavage. The curves then level off, but *A* gradually approaches *B*. The 1 per cent extract of *Daphnia* decreased 84△V in 20 minutes while a 1 per cent extract of hog pancreas fell to 100△V in the same time.

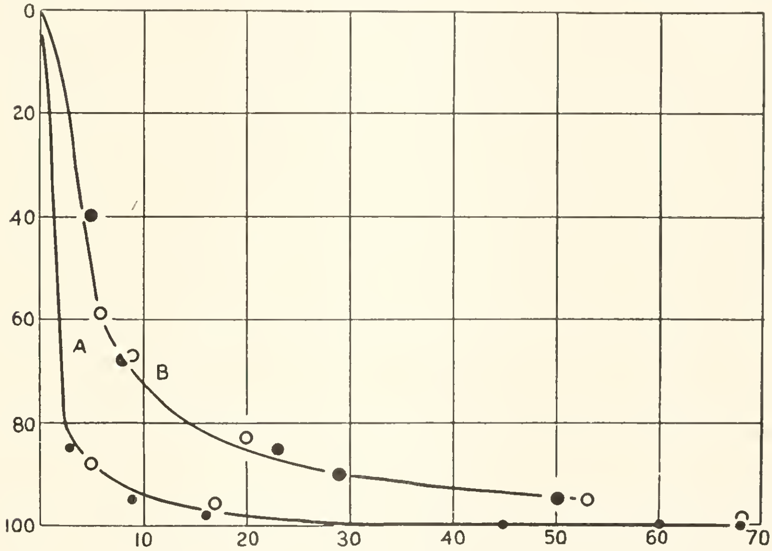


Fig. 1. Hydrolysis of gelatin at pH 7.4 by proteinases of hog pancreas (*A*), and of *Daphnia* (*B*). The ordinate represents decreasing viscosity (△V); the abscissa is the time in minutes.

EFFECT OF pH ON ENZYME ACTIVITY

The character of an enzyme is determined by the pH of optimum activity. Mammalian peptic enzymes are most active at pH 1.0, tryptic enzymes pH 7–8 and katheptic enzymes pH 4–5. The subsequent procedure was followed in determining the effect of pH upon proteinase activity of the *Daphnia* extracts. The 1 per cent extract used in the above experiment was diluted to contain 1.5 *Daphnia* proteinase units. A unit was defined as the amount of enzyme necessary to cause a decrease of 20△V of 1.5 per cent Sargent's gelatin in 20 minutes; pH 7.4, temperature 34° C.

Test tubes of 1.5 per cent Sargent's gelatin were buffered in a series at hydrogen-ion concentrations of 1–10 and preserved with thymol. The pH was determined with the aid of the quinhydrone electrode. To 5 cc. of gelatin at a desired pH, 0.5 cc. of enzyme solution containing 1.5 units was added. Viscosity readings were made and a graph constructed for proteinase activity. The readings at the end of 50 minutes, for each of the pH 1–10 runs, were taken and plotted against pH. The activity of the proteinase at any pH can be read from Fig. 2. The optimum activity was reached at pH 7.4. Its activity decreased on the alkaline side of pH 7.4. On the acid side, the enzyme was found to be inactive at pH 3.2. The graph was constructed from the mean of two runs at each pH. A control was used in all cases and the experiment duplicated three times.

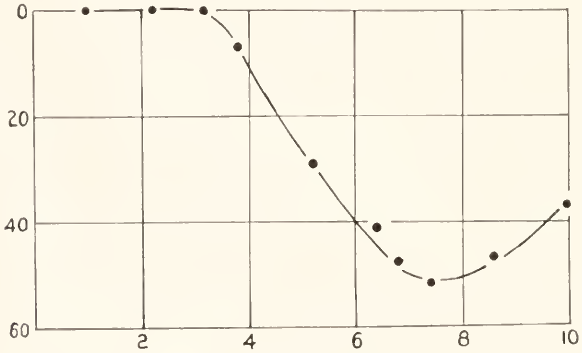


FIG. 2. Activity of *Daphnia* proteinases—effect of pH on gelatin hydrolysis. Decreasing viscosity (△V) is the ordinate; the abscissa represents pH.

AMYLASE

The amount of maltose produced by the action of 1 per cent extract on a 3 per cent corn starch solution was iodometrically titrated by the

Baker and Hulton (1920) method for the estimation of sugars and used as an indication of amylase activity. A 10-cc. sample starch solution, pH 7.4, was diluted to 50 cc. and used as the substrate. To this was added 1 cc. of extract and thymol for preservative, and it was then kept at 37° C. Every 15 minutes 5-cc. samples were withdrawn and titrated. The increase in the amount of thiosulphate used in the titration was equivalent to the same amount of maltose liberated in the digest. Duplicate samples were used. The curves in Fig. 3 represent the mean of two runs. The control showed no hydrolysis during the run.

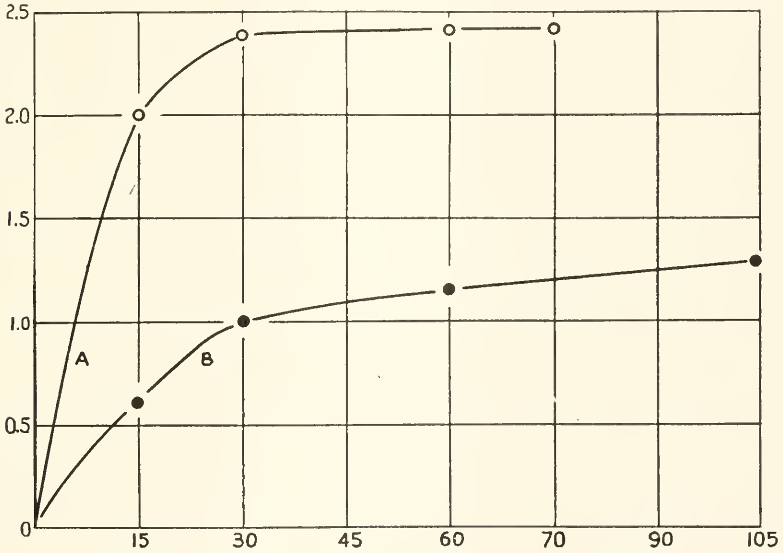


FIG. 3. Hydrolysis of starch at pH 7.4 by amylase of hog pancreas (*A*), and of *Daphnia* (*B*). The ordinate represents the number of cc. of N/20 maltose; the abscissa is the time in minutes.

The concentration of amylase is much greater in the glycerol extract of hog pancreas than in the *Daphnia*. After 30 minutes the hydrolysis of the starch by 1 per cent hog pancreas extract was equivalent to 2.4 cc. of N/20 maltose, while in the same time 1.0 cc. was the total amount produced by the *Daphnia* extract.

LIPASE

For the determination of lipase activity, 50 cc. of 4 per cent tributerine were emulsified with sodium glycocholate. To this were added 2 cc. of 1 per cent extract. The digest was placed at 37° C. and 10-cc. samples were withdrawn hourly and titrated with N/20 NaOH. The increase in acidity due to the liberation of fatty acids by the hydrolytic action of the lipase in the *Daphnia* extract and hog pancreas extract was recorded in terms of N/20 NaOH. The results are shown in Fig. 4. The experiments were duplicated and run with controls. The presence of a lipase in the *Daphnia* extract is clearly demonstrated. The mean acidity at the end of four hours was equivalent to 0.9 cc. of N/20 NaOH in the case of 1 per cent glycerol extract of *Daphnia* and 2.6 for hog pancreas.

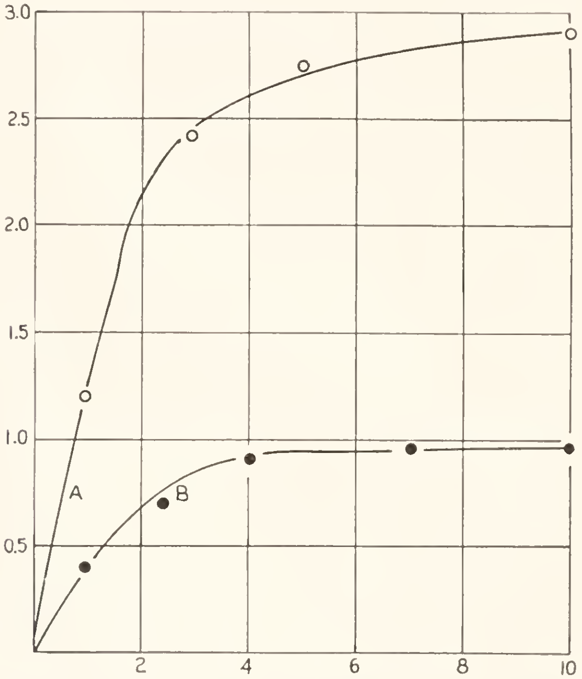


FIG. 4. Hydrolysis of tributerine by lipase of hog pancreas (*A*), and of *Daphnia* (*B*). The ordinate represents the number of cc. of N/20 NaOH; the abscissa is the time in hours.

DISCUSSION

The experiments show the presence of a proteinase, amylase, and lipase which partially complete the enzyme mechanism for handling proteins, carbohydrates, and fats available to *Daphnia* in its natural habitat and under natural feeding conditions. These experiments show that the activities of the proteinase and amylase are such that considerable digestion is possible in the 15–30 minutes that food is said to remain in the alimentary tract. From Fig. 1 it was computed that 88 per cent of the total digestion of gelatin done by a 1 per cent extract of *Daphnia* was completed within 20 minutes. Fig. 3 shows that of the total amount of maltose formed by amylase in 75 minutes, 80 per cent was produced in the first 30 minutes.

Figure 1 illustrates a comparison of the proteolytic activity of a 1 per cent glycerol extract of defatted *D. pulex* and a 1 per cent glycerol extract of defatted hog pancreas. It is apparent that the hog pancreas extract contains more enzyme than the *Daphnia* extract, due primarily to the fact that the digestive enzymes of the hog are most concentrated in the pancreas. On the other hand, however, if the amount of proteinase present per body weight of *Daphnia* be compared with the amount per body weight of hog, the picture would look very different. Estimating a hog to weigh 200 lbs., the hog pancreas 0.5 lbs., and the average *Daphnia* 0.0043 oz., rough calculations approximate the amount of proteinase to be from 300 to 400 times greater per body weight of *Daphnia* than per body weight of hog.

The proteinase obtained by gross extraction of the entire organism is found to have an optimum digestion at pH 7.4. This extract may obviously contain proteases other than the digestive enzymes, and any interpretation of the results may be made with this in view. Isolation of a proteinase from the digestive tract of *Daphnia* was not attempted. On the other hand, the extracts of 50 isolated digestive tracts of *D. magna* showed definite proteolytic activity at pH 7, which corresponds with the pH observed in the digestive tract and with the optimum pH of the enzyme mixture obtained from the entire animal. It will be recalled that mammalian tissue proteases react best in slightly acid media-about pH 4 +, and not at all at 7. The author believes, therefore, that he is warranted in assuming that the proteinase obtained by extracting the entire *Daphnia,* acting best at pH 7.4, is the digestive enzyme of the alimentary tract.

The character of the proteinase simulates that of mammalian trypsin and the initial cleavages on gelatin were undoubtedly produced by this tryptic-like proteinase, since Waldschmidt-Leitz (1929) holds that erepsin does not hydrolyze gelatin. It is very probable, however, that erepsin was present and played some part in the splitting of the poly- and di-peptides after the initial cleavages by the proteinase had been made. In addition, the fact that the extract was practically inactive at pH 3.8 and completely inactive at 3.2 rules out the possibility of the presence of a peptic type of digestion.

SUMMARY

1. A digestive enzyme system exists in *Daphnia* that enables it to digest considerable protein and carbohydrate within 30 minutes.

2. Microchemical analysis of glycerol extracts of *D. magna* intestines demonstrated the presence of a proteinase similar to trypsin. Quantitative estimations were made of proteinase activity of whole *D. pulex* extracts.

3. No pepsin was found in the extracts.

4. Amylase and lipase were chemically demonstrated in extracts of *Daphnia*.

5. The pH of the alimentary tract of *D. magna* was found to range from 6.8–7.2.

LITERATURE CITED

Baker, J., and H. F. E. Hulton, 1920. The Iodimetric Estimation of Sugars. *Biochem. Jour.*, **14**: 754.

Birge, E. A., and C. Juday, 1922. The Inland Lakes of Wisconsin. The plankton. 1. Its quantity and chemical composition. Bull. No. 64. Wis. Geol. and Nat. Hist. Survey.

Birge, E. A., and C. Juday, 1934. Particulate and Dissolved Organic Matter in Inland Lakes. *Ecol. Mon.*, **4**: 440.

Gates, F. L., 1927. A Method of Proteolytic Enzyme Titration. *Proc. Soc. Exper. Biol. and Med.*, **24**: 936.

Klugh, A. B., 1927. The Ecology, Food-Relations and Culture of Fresh-Water Entomostraca. *Trans. Roy. Can. Inst.*, **16**: 15.

Krogh, A., 1930. Über die Bedeutung von gelösten organischen Substanzen bei der Ernährung von Wassertieren. *Zeitschr. vergl. Physiol.*, **12**: 668.

Naumann, E., 1918. Über die natürliche Nahrung des limnischen Zooplanktons. *Lunds Univ. Arsskrift* N.F. Avd. 2, **14** (No. 31): 1.

Naumann, E., 1921. Spezielle Untersuchungen über die Ernährungsbiologie des tierischen Limnoplanktons. 1. Über die Technik des Nahrungserwerbs bei den Cladoceren und ihre Bedeutung für die Biologie der Gewässertypen. *Lunds Univ. Arsskrift.* N. F. Avd. 2, **17** (No. 4): 1.

Northrop, J. H., and R. G. Hussey, 1922. A Method for the Quantitative Determination of Trypsin and Pepsin. *Jour. Gen. Physiol.*, **5**: 353.

Pütter, A., 1909. Die Ernährung der Wassertiere und der Stoffhaushalt der Gewässer. Gustav Fischer. Jena.

Rankin, G. P.,1929. The Nutritional Physiology of Cladocera. *Cont. Canad. Biol. and Fish.*, **4** (No. 8): 109.

Stuart, C. A., M. McPherson, and H. J. Cooper, 1931. Studies on Bacteriologically Sterile Moina macrocopa and their Food Requirements. *Physiol. Zool.*, **4**: 87.

Waldschmidt-Leitz, E., 1929. Enzyme Actions and Properties, p. 147. John Wiley and Sons.

THE EFFECT OF CUPRIC, MANGANOUS, AND FERRIC CHLORIDES UPON CARDIAC EXPLANTS IN TISSUE CULTURE

DUNCAN C. HETHERINGTON AND MARY E. SHIPP

(From the Department of Anatomy, Duke University School of Medicine)

INTRODUCTION

Considerable material has appeared in the literature concerning the effect of many metallic ions and salts upon various biological functions. It has appeared from the studies of Titus and Cave (1928), Titus, Cave and Hughes (1928) and Elvehjem (1932) that manganese and iron are particularly prominent in numerous rôles, especially in cellular respiration, hemoglobin formation, and nutrition. To introduce a third element, copper likewise is essential to hemoglobin formation regardless of the amount of iron administered to the organism (Titus and Cave, 1928). Further, it was shown by Titus and Hughes (1929) that copper and manganese may be stored in the animal body in such a way as to be effective in the utilization of iron in respiratory pigment formation. Later studies by Locke and Main (1931) have provided additional evidence that iron is essential for the regulation of cellular respiration and that it, together with copper, forms the oxygen-binding nucleus of the respiratory pigments. The absorption of oxygen by the cells is due to a reaction between molecular oxygen and a complex intracellular iron compound which is believed to be a hematin derivative and is termed "the respiratory enzyme" (Warburg, 1923*a*, 1923*b*, 1925, 1926, 1928; Warburg, Posener and Negelein, 1924).

The fact that these metals play a further part in the proper function of the blood is brought out by Meyer and Eggert (1932), who reported beneficial results following the administration of copper and iron combinations in the treatment of secondary anemia, while the liver and liver extracts used in primary anemia have appeared to be effective for reasons other than that they contain high percentages of these metals. On the other hand, the beneficial effects of hepatic therapy in secondary anemia may be due, at least in part, to the presence of these two metals.

The absence of iron and copper from synthetic media results in the slowing up of yeast cell proliferation and the production of atypical cells with low pigment content. This reduction of pigment has been held to be comparable, in certain respects, to the condition of anemia in animals (Elvehjem, 1931).

Leaving the question of iron and copper for the moment and turning to that of manganese, it has been shown that horses and goats, immunized against diphtheria, and exhibiting a constant fall of antitoxin titre, would increase the titre following injections of manganese chloride. Small injections of this salt increased the power of the organism to destroy the bacterial toxins to such a degree that animals so treated were not poisoned by an otherwise fatal dose of toxin. This was particularly true in cases of tuberculosis in mice and guinea pigs (Walbum, 1921, 1924). Although manganese will raise the titre of diphtheria antitoxin, it will not change that of tetanus, nor has it any effect upon agglutinins or hemolysins (Pico, 1924).

The experiments reported in the present article were devised to test the effect of different concentrations of the three metals mentioned upon the growth and longevity of cells in tissue cultures. The 3,000 cultures examined in this study have yielded some rather definite information on the questions of the toxicity of, and the tolerance to, these elements when used singly and in combinations. It became of interest to determine whether a tolerance could be built up for such solutions as proved toxic; and whether a combination of non-toxic, or even beneficial solutions was more beneficial than the constituents used separately.

TECHNIQUE

All of the cultures used in these experiments were made by the cover-slip-hanging-drop method. The procedures were carried out with aseptic precautions in each instance. All water used was triply distilled in an all-Pyrex-glass apparatus. The plasma was obtained by centrifuging blood drawn from the wing veins of young hens. Embryo juice (33 per cent) was prepared by extracting seven-day, or eight-day chick embryos in Tyrode solution (pH 7.4 to 7.6) containing 0.25 per cent dextrose. In order to increase the potency of the extract, it was allowed to stand twenty-four hours at 40° F. before centrifuging (Carrel, 1913). In the experimental series the metallic salts (Merck's), in sufficient quantities for the proper final dilutions, were added to the embryo juice before mixing. Tissue from the hearts of seven-day, or eight-day chick embryos was planted in a mixture of equal quantities of plasma and embryo juice containing the metallic salt. An equal number of controls, using the same plasma, stock embryo juice, and embryo heart tissue, was run with each series of the metallic chloride cultures. These cultures were incubated at 39° C. and careful daily records were kept on each as long as it remained alive. An average percentage death curve was constructed from the data obtained in the control cultures of all experiments in this paper and serves as the standard for comparison with the curves obtained from the metallic ion series.

EXPERIMENTAL

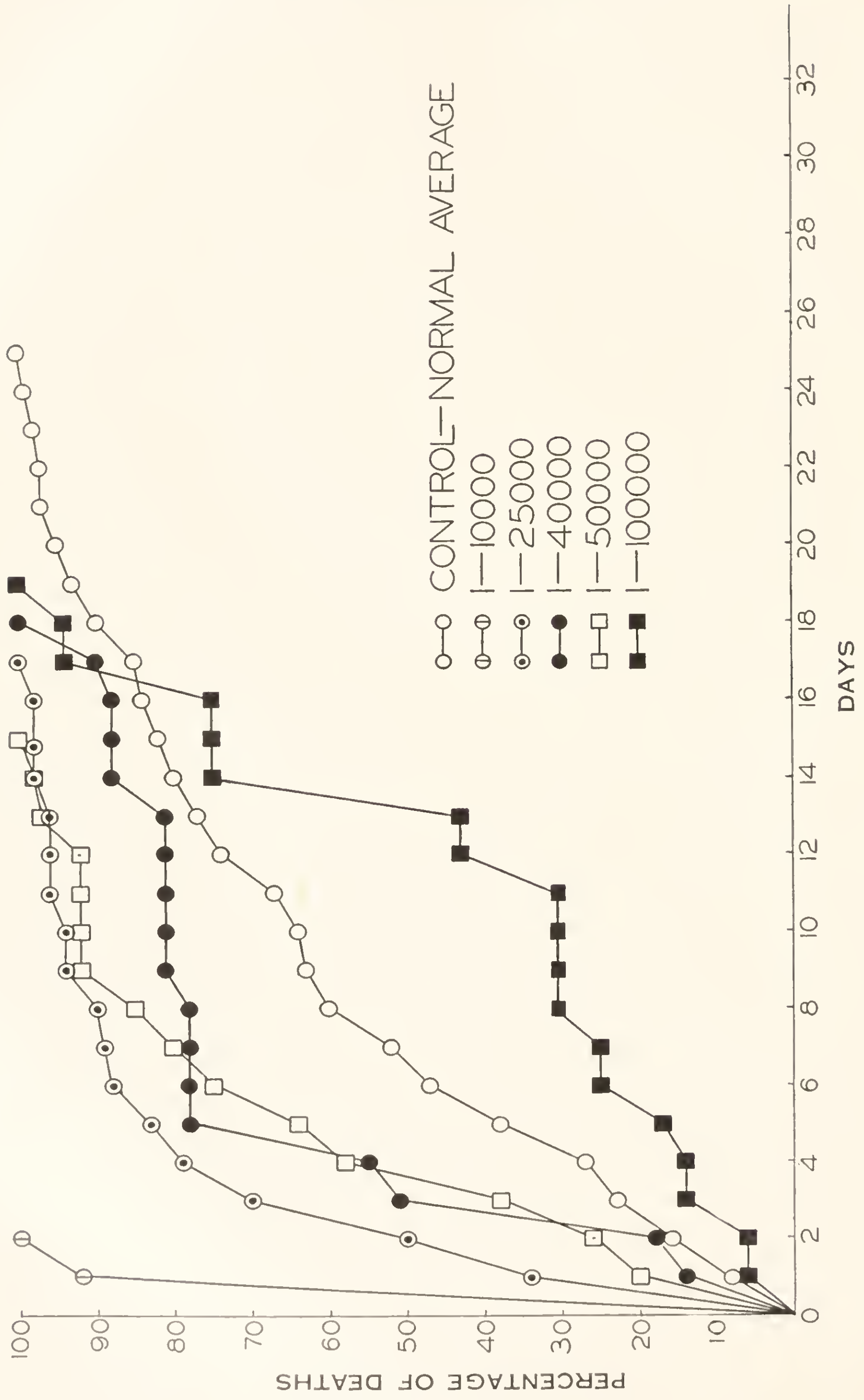
Cupric Chloride Experiments

In these experiments to test the effect of the copper salt, $CuCl_2 .2H_2O$ was added to embryo juice samples in sufficient quantities to make a range of dilutions and normalities, after an equal quantity of plasma had been added, as follows: 1–10,000 (.00117 N), 1–25,000 (.00056 N), 1–40,000 (.00029 N), 1–50,000 (.00023 N), 1–100,000 (.000117 N).

The average death curve of the control cultures climbed rapidly at first and then more gradually until the twenty-fifth day when all cultures were dead. Cupric chloride—1–10,000 (Graph I)—was found to be very toxic to the explants, as evidenced by an almost straight curve; 92 per cent of all the cultures died within twenty-four hours and the remaining 8 per cent within forty-eight hours. In cultures with this concentration of copper, growth was practically inhibited; only occasionally was there any evidence of the migration of fibroblasts. In the 1–25,000 dilution series a fair radial outgrowth of mesenchymal cells was present twenty-four hours after planting. However, the curve rises very rapidly until the sixth day, after which it resembles the control curve. This may be due to either of two factors or a combination of them. It is possible that the tissues became accustomed to the copper salt or, after active proliferation of fibroblasts began, the toxicity of the chloride may have become proportionately less as the cell mass increased.

The curves of cultures planted in 1–40,000 and 1–50,000 dilutions of the copper salt rise rapidly and more or less in company until the fifth day. Then they diverge and the lesser dilution curve flattens out, rising slowly until the seventeenth day. On the other hand, the 1–50,000 dilution curve continues to rise until the ninth day, after which the rise is less acute until the fifteenth day when all cultures were dead. The radial outgrowth of cells in both these dilutions was good, though markedly better in the greater dilution. This fact may account for the different nature of the curve. The possibility, which suggests itself here, is that the life span was shorter because of lack of nutriment.

In the 1–100,000 cupric chloride series, the radial outgrowth of the cells was very marked and exceeded the growth of the controls. This is evidenced by the curve which shows that on the eleventh day only 30.5 per cent of the cultures were dead as compared with 67 per cent of the controls. However, from this point on, the curve rises very



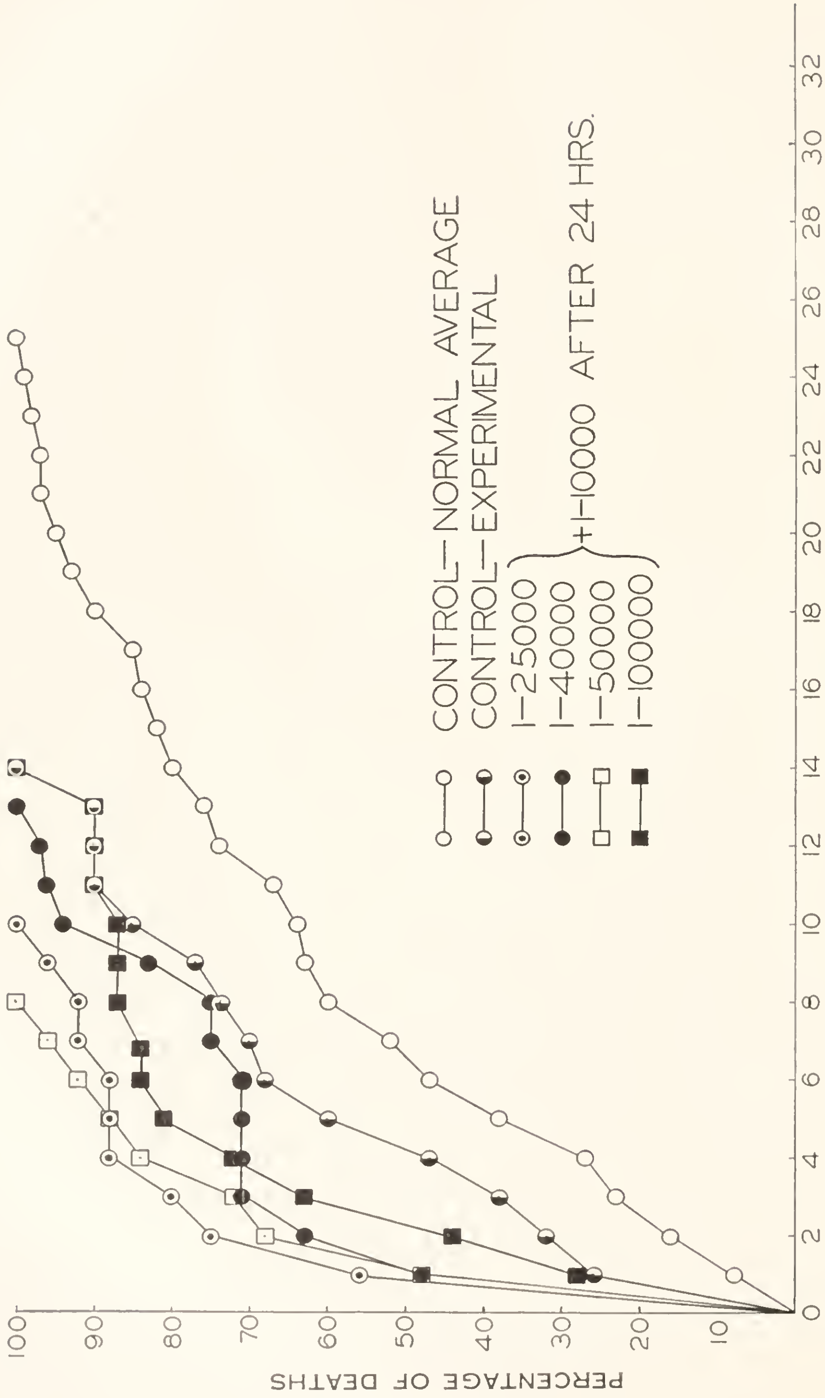
Graph I. Cupric chloride dilutions.

rapidly, possibly because of the toxic effect of the salt together with the lack of nutriment and accumulation of waste products.

A second series of experiments to test a possible tolerance of the cells to cupric chloride was carried out by the administration of a toxic concentration of the metallic salt after a period of adjustment in greater dilutions. Cultures were planted in each of the four salt dilutions: 1–25,000, 1–40,000, 1–50,000 and 1–100,000 and allowed to incubate for twenty-four hours. At the end of this period of preliminary adjustment and growth, each living culture was unsealed, the fluid drawn off and replaced by one drop of 1–10,000 copper chloride embryo juice. This concentration was selected because it had been found to be the most toxic of the solutions employed. Following this treatment the cultures were sealed, incubated as usual, and observed at twenty-four hour intervals until all were dead. The experimental controls for this series consisted of cultures planted in the regular manner without any copper salt added until after the first twenty-four hours of incubation when one drop of 1–10,000 copper chloride embryo juice was added. Thereafter the results were recorded as for the other series and all appear in Graph II.

For the first three days the percentage death curves have much the same shape, rising rapidly. During this interval the toxicity was most marked in the 1–25,000 dilution (80 per cent), about the same for 1–40,000 and 1–50,000 (71 and 72 per cent), less in the 1–100,000 (63 per cent) and least in the experimental control (38 per cent). If one compares the curve of cultures planted in 1–10,000 $CuCl_2$ (Graph I) with the curves of the culture series in the varying dilutions to which 1–10,000 $CuCl_2$ was added after twenty-four hours (Graph II), one notes that the additional copper is tolerated rather well though the life span is much less (a difference of eleven days for 1–100,000 $CuCl_2$ series) for all series compared with that of the normal controls. The experimental controls, which were normal cultures allowed twenty-four hours undisturbed growth and then subjected to copper chloride 1–10,000, died off less rapidly than the cultures planted in the various dilutions and then treated with the toxic dose. The total life span, however, was the same as that of the 1–100,000 dilution cultures in this particular experiment. It seems from this that a very critical period in the life of the explanted tissues occurs within the first twenty-four hours of adjustment and growth.

Another experiment was devised to test whether the age of the culture, before adding the toxic 1–10,000 $CuCl_2$, would influence the tolerance as evidenced by the longevity of the tissue. A large series of cultures was planted in the various dilutions of copper chloride as before



Graph II. Cupric chloride tolerance.

and divided into groups. After twenty-four hours incubation a set of vigorous cultures from each dilution series was unsealed and each preparation received one drop of 1–10,000 $CuCl_2$ embryo juice. They were resealed and returned to the incubator. On the second, third, fourth and fifth days a fresh group from each series was similarly treated. By this means the cultures were allowed to establish growths in their respective copper dilutions from one to five days before their susceptibility to the toxic concentration of copper was tested.

The results of this experiment were rather unsatisfactory. However, the series in which the procedure duplicated that of the tolerance experiment just described, i.e., addition of the toxic solution on the day following planting, reproduced the curves of Graph II almost exactly. Two days difference in the life span was the greatest variation and that occurred in the 1–50,000 dilution.

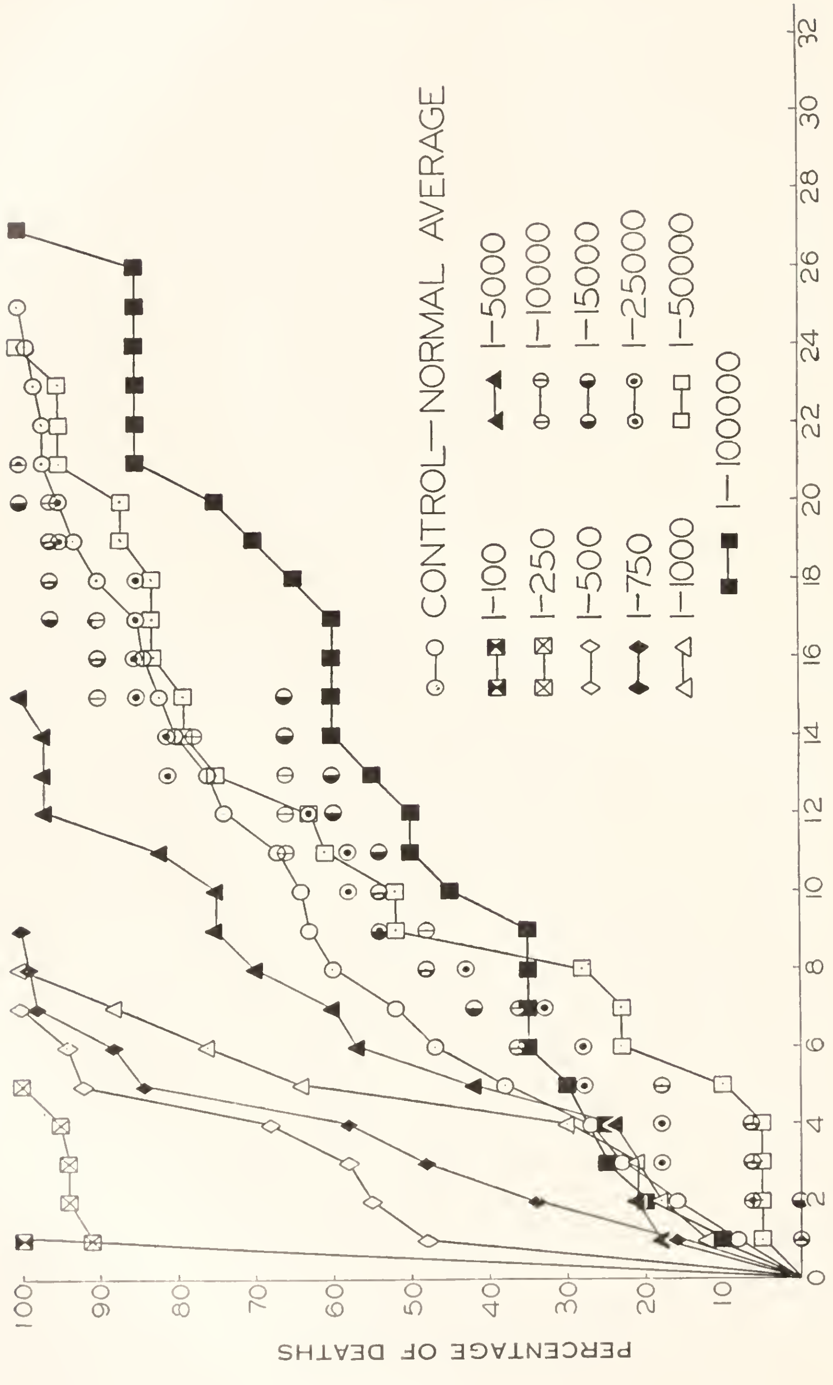
With the exception of the cultures in 1–25,000 dilution, the optimum resistance to an added toxic dose of copper salt, as evidenced by plotting the results, was acquired between the third and fourth days of growth.

Manganous Chloride Experiments

Experiments, conducted in a manner similar to those of the copper series, were carried out with manganous chloride, $MnCl_2.4\ H_2O$, added to the embryo juice. This salt was added in quantities sufficient to form the following final dilutions in which series of cultures were planted: 1–100 (.10 N), 1–250 (.04 N), 1–500 (.02 N), 1–750 (.013 N), 1–1,000 (.010 N), 1–5,000 (.002 N); 1–10,000 (.0001 N), 1–15,000 (.0006 N), 1–25,000 (.0004 N), 1–50,000 (.0002 N), 1–100,000 (.0001 N).

When the percentage death curves were plotted and compared with the average curve of all normal controls, it was found (Graph III) that they fell roughly into three major groupings. The series 1–100 through 1–1,000 showed a marked toxicity of the salt; the 1–5,000 sets were isolated midway between these and the remaining dilutions. The curves of all dilutions from 1–15,000 through 1–50,000 remained below the control curve until the twelfth day after which they fell to one side or the other of this curve. The 1–100,000 dilution curve, from the fourth day onward, was always below that of the control and terminated on the twenty-seventh day, exceeding the control in length by two days.

From these curves it will be seen that the presence of manganous chloride in dilutions of 1–15,000 through 1–50,000 did not markedly influence the behavior of cells in cultures although the life span was decreased one to five days. The cells grew and radial outgrowth was



Graph III. Manganous chloride dilutions.

almost as prolific as in the controls The aging process,[1] slower in these cultures than in the controls, was hastened a little after the twelfth day.

Manganous chloride 1–100,000 prolonged the life of cultures for two days as compared with the controls. This in itself was not a significant increase, but in view of the fact that the percentage deaths throughout the series on any one day after the sixth were from 10 to 20 per cent less than for the normal controls, it seemed that manganese in this concentration may have exerted a slight influence in the direction of longevity.

To ascertain whether the cultures would develop a tolerance for manganese, a series of plantings in 1–100,000 dilution were allowed to adjust themselves for twenty-four hours. At the end of that time they were unsealed and to each culture was added one drop of 1–100 $MnCl_2$ embryo juice. Then they were resealed and returned to the incubator. The results of these series showed that the addition of the very toxic solution (see Graph IV) did not cause the death curve to rise precipitously as in the 1–100 dilution series, but rather to rise rapidly until the tenth day and thereafter to follow very closely the curve of the 1–50,000 dilution. It would seem, therefore, that preliminary treatment with a non-toxic manganese concentration protected, to some extent, cultures subjected later to a fatal concentration.

Ferric Chloride Experiments

In a manner similar to that used in previous experiments plantings were made in the following $FeCl_3.6\ H_2O$ solutions: 1–250 (.044 N), 1–500 (.022 N), 1–1,000 (.011 N), 1–10,000 (.0011 N), 1–50,000 (.00022 N), 1–100,000 (.00011 N).

The results of these series are shown in Graph V. The dilution 1–250 proved to be highly toxic, 90 per cent of the cultures died in the first twenty-four hours and all of them were dead in five days. The curves of the other dilutions except 1–500 and 1–1,000 followed quite closely that of the average normal control, falling but a short distance above or below it until the eighteenth day when they diverged. With the exception of the 1–250 dilution, all other curves exceeded the normal in duration from three to eight days. Contrary to the results obtained for the copper and manganese chlorides, the 1–1,000 dilution of the $FeCl_3$ proved to be the least toxic of all and the life span of this series was prolonged eight days over that of the controls. This is a significant increase and might suggest that this dilution of ferric chloride was

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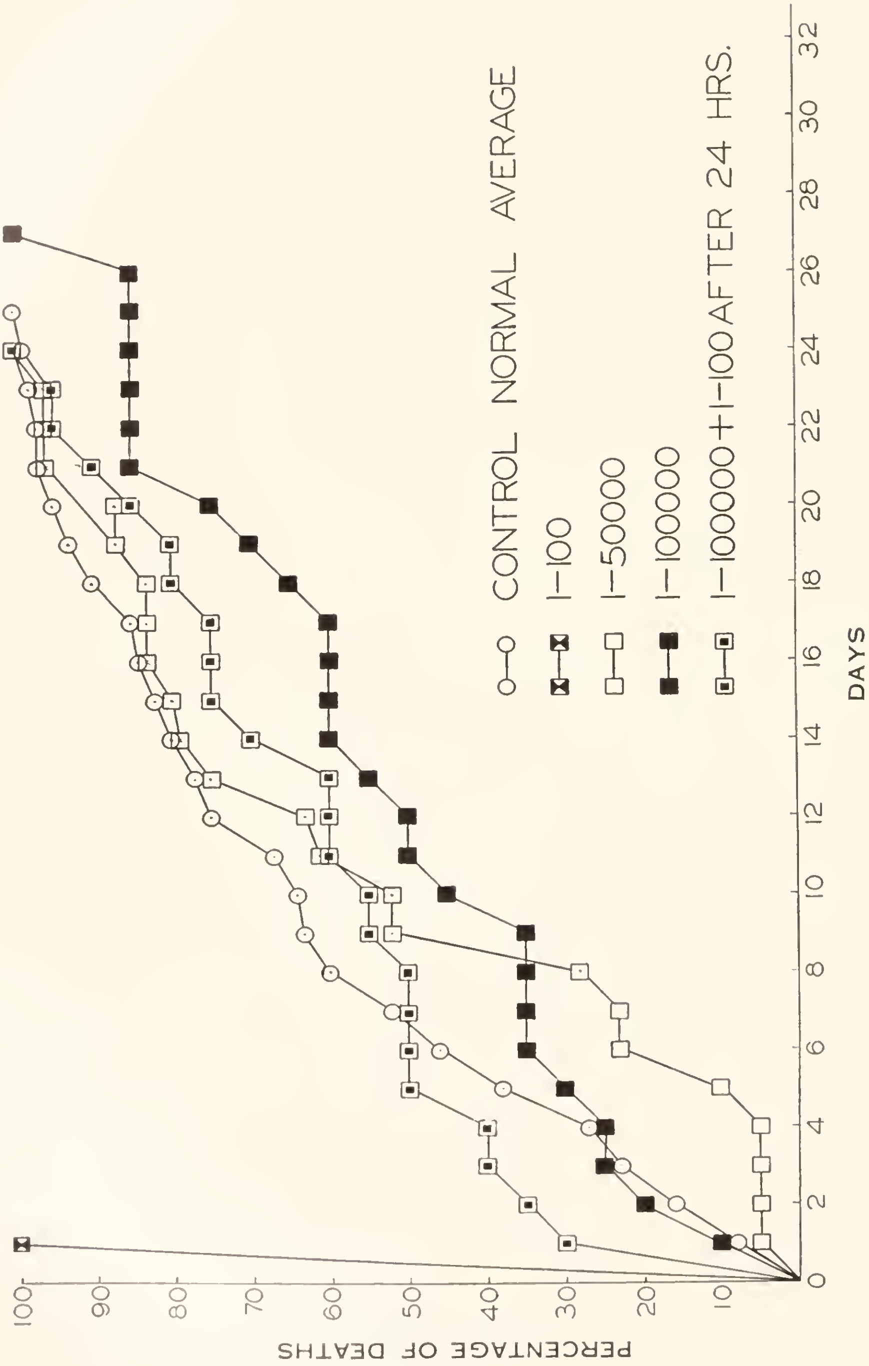
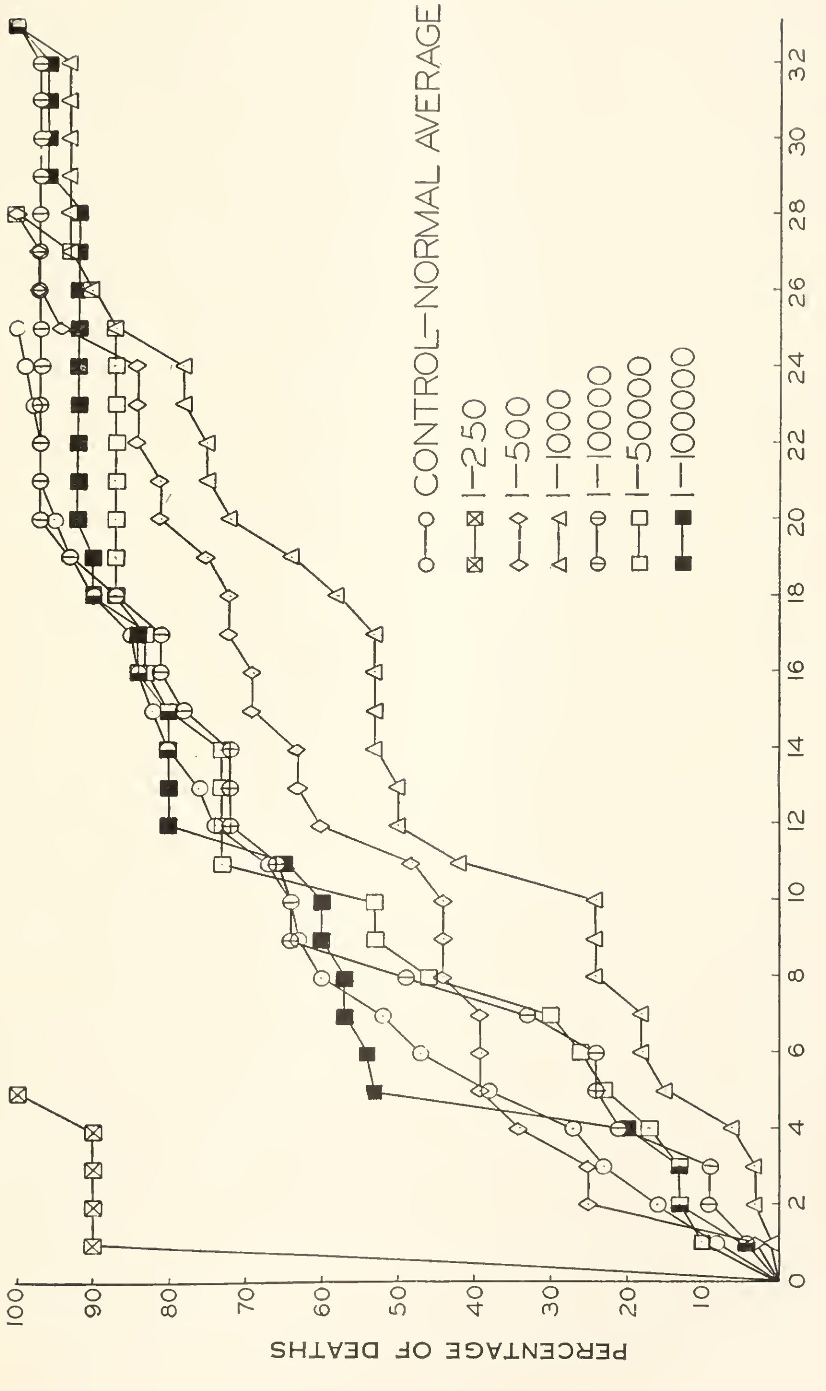
[1] The addition of potassium permanganate to mesenchymal cells in cultures was found by Lewis (1921) to reproduce, within a few minutes, the degenerative changes that took place gradually in the normal aging and resultant death of cells.



Graph IV. Manganous chloride tolerance.



Graph V. Ferric chloride dilutions.

beneficial to cultures. However, one observation must be kept in mind in this connection. After tissues were planted in the $FeCl_3$ solutions, a brown precipitate was produced in the medium and in all the iron salt cultures, histiocytes were found in noticeably greater numbers than in either copper or manganese cultures. These phagocytic cells contained quantities of engulfed iron precipitates. Whether the formation of precipitates rendered the iron less toxic or whether the abundance of histiocytes exerted a beneficial influence upon the cultures is not known. As suggestive evidence, only, in support of the latter idea, the senior author noted, in some other experiments using cardiac tissue planted in a medium containing trypan blue, that histiocytic outgrowth was stimulated and that cultures so populated remained in better condition longer than those without an abundance of macrophages.

In order to test the development of tolerance to iron, a series of cultures was planted in 1–1,000 $FeCl_3$ and incubated for twenty-four hours before each culture received one drop of 1–250 iron salt. As in previous experiments of this nature, the preliminary treatment of the cultures with the least toxic dilution of the salt lessened (Graph VI) to a marked degree the toxicity of a fatal dose; although the life span was reduced from thirty-three days (1–1,000 dilution curve) to twenty-six days (tolerance curve) as compared with the five-day span for cultures planted only in 1–250 dilution.

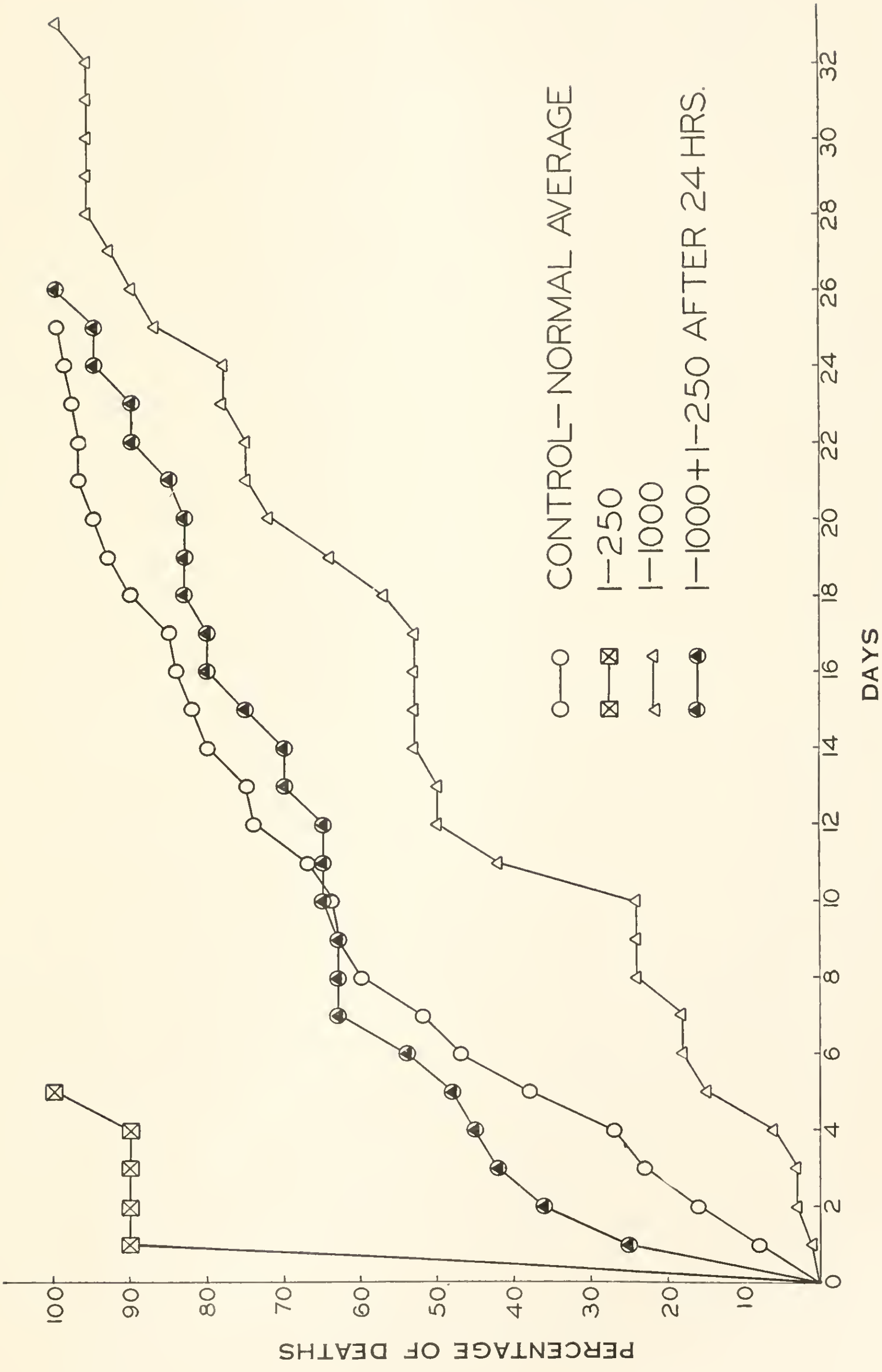
Combination Experiments

In view of the fact that certain dilutions of each of the three metallic chlorides utilized in the preceding experiments were found to be relatively non-toxic, four combinations of the optimum dilutions of the different chlorides were made and cultures were planted in them. The same technique used in the previous experiments was employed here.

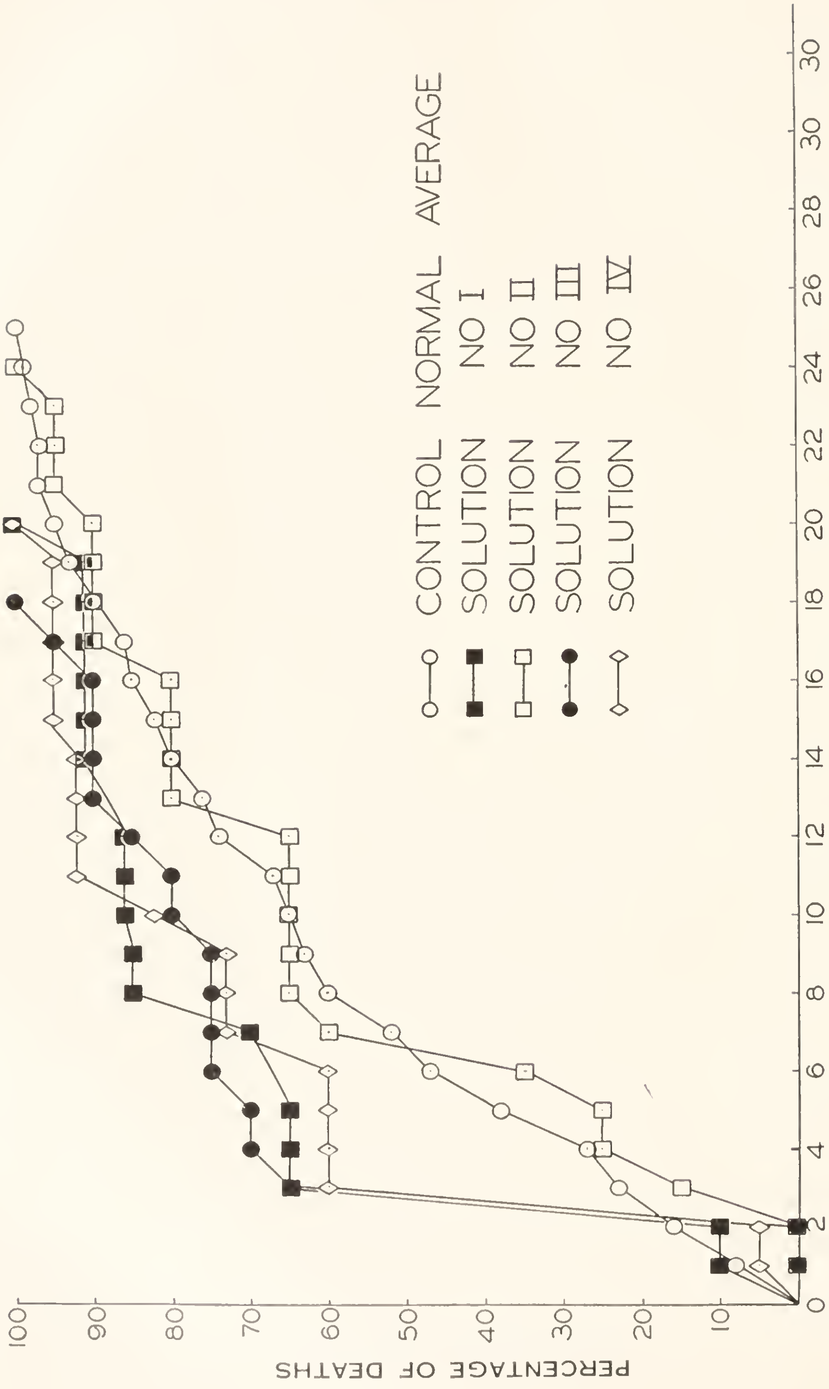
The salt mixtures used were as follows:

Solution 1.	(*a*) Ferric Chloride	1–1,000
	(*b*) Cupric Chloride	1–100,000
	(*c*) Manganous Chloride	1–100,000
Solution 2.	(*a*) Ferric Chloride	1–1,000
	(*b*) Manganous Chloride	1–100,000
Solution 3.	(*a*) Ferric Chloride	1–1,000
	(*b*) Cupric Chloride	1–100,000
Solution 4.	(*a*) Manganous Chloride	1–100,000
	(*b*) Cupric Chloride	1–100,000

Solutions 1, 3, and 4 were the most toxic and caused sudden death of 60 to 65 per cent of the cultures by the third day (Graph VII); those remaining died by the eighteenth to twentieth days as compared with a life span of twenty-five days for the normal controls. Solution 2 was



Graph VI. Ferric chloride tolerance.



Graph VII. Combination solutions.

the least deleterious; its curve followed closely that of the control and the life span of the series was only one day less. None of the salt mixtures had any advantage over the single optimum dilutions of the respective salts composing it. It was noted again, however, that whereever iron was present in the solutions, the cultures contained a greater number of histiocytes than otherwise.

SUMMARY

Throughout these experiments there were certain characteristic reactions in the mesenchymal cells growing in the various dilutions of the metallic chlorides used. The nuclear membrane and nucleoli were generally a little more sharply defined than in the cells of the controls. Accumulation of vacuoles, first about the nucleus and finally throughout the cytoplasm, took place rapidly in the toxic salt solutions. At times the cells were so thoroughly occupied by fatty looking vacuoles that they bulged, rounded up and floated away. Similar accumulations of vacuoles occurred in the controls but their appearance was gradual and they only assumed great numbers as the cultures aged.

Optimum dilutions of the metallic salts, in which a depression of the daily death rate and an increase in the life span of the cultures were noted, delayed the degenerative changes in the cells beyond the time when they usually made their appearance in the controls.

Of these solutions, cellular outgrowth was actually stimulated in that of copper chloride, the death rate was depressed in that of manganese chloride, as also in that of iron chloride. Furthermore, the life span was increased in the latter a significant number of days. This effect possibly may have been brought about indirectly as a result of histiocytic stimulation.

Tissues could be protected to some extent from the action of a fatal dose of these three metallic salts by first growing the cells in the respective optimum dilutions.

No beneficial effects were obtained by growing tissues in various combinations of the optimum dilutions of these salts.

LITERATURE CITED

CARREL, A., 1913. Artificial Activation of the Growth in Vitro of Connective Tissue. *Jour. Exper. Med.*, **17**: 14.

ELVEHJEM, C. A., 1931. The Rôle of Iron and Copper in the Growth and Metabolism of Yeast. *Jour. Biol. Chem.*, **90**: 111.

ELVEHJEM, C. A., 1932. The Relative Value of Inorganic and Organic Iron in Hemoglobin Formation. *Jour. Am. Med. Ass.*, **98**: 1047.

LEWIS, WARREN H., 1921. The Effect of Potassium Permanganate on the Mesenchyme Cells of Tissue Cultures. *Am. Jour. Anat.*, **28**: 431.

Locke, A., and E. R. Main, 1931. The Relation of Copper and Iron to Production of Toxin and Enzyme Action. *Jour. Infect. Dis.*, **48**: 419.

Meyer, A. E., and C. Eggert, 1932. Iron and Copper in Liver and Liver Extracts. *Jour. Biol. Chem.*, **99**: 265.

Pico, C. E., 1924. Influence du manganèse sur les phénomènes de l'immunité. *Compt. rend. Soc. Biol.*, **91**: 1049.

Titus, R. W., and H. W. Cave, 1928. Manganese as a Factor in Hemoglobin Building. *Science*, **68**: 410.

Titus, R. W., H. W. Cave, and J. S. Hughes, 1928. The Manganese-copper-iron Complex as a Factor in Hemoglobin Building. *Jour. Biol. Chem.*, **80**: 565.

Titus, R. W., and J. S. Hughes, 1929. The Storage of Manganese and Copper in the Animal Body and Its Influence on Hemoglobin Building. *Jour. Biol. Chem.*, **83**: 463.

Walbum, L. E., 1921. Action exercée par le chlorure de manganèse et d'autres sels métalliques sur la formation de l'antitoxine diphtérique et l'agglutinine du B. coli. *Compt. rend. Soc. Biol.*, **85**: 761.

Walbum, L. E., 1924. Therapeutic Experiments with Metal Salts. *Acta pat. et microbiol. Scandinav.*, **1**: 378.

Warburg, O., 1923*a*. Versuche an überlebendem Carcinomgewebe. *Biochem. Zeitschr.*, **142**: 317.

Warburg, O., 1923*b*. Über die Grundlagen der Wielandschen Atmungstheorie. *Biochem. Zeitschr.*, **142**: 518.

Warburg, O., 1925. Bemerkung zu einer Arbeit von M. Dixon und S. Thurlow sowie zu einer Arbeit von G. Ahlgren. *Biochem. Zeitschr.*, **163**: 252.

Warburg, O., 1926. Über die Wirkung von Blausäureäthylester (Äthylcarbylamin) auf die Pasteursche Reaktion. *Biochem. Zeitschr.*, **172**: 432.

Warburg, O., 1928. Wie viele Atmungsfermente gibt es? *Biochem. Zeitschr.*, **201**: 486.

Warburg, O., K. Posener, and E. Negelein, 1924. Über den Stoffwechsel der Carcinomzelle. *Biochem. Zeitschr.*, **152**: 309.

THE NUTRITION OF COPEPODS IN RELATION TO THE FOOD-CYCLE OF THE SEA

G. L. CLARKE AND S. S. GELLIS

(From the Woods Hole Oceanographic Institution[1] *and the Biological Laboratories, Harvard University)*

The importance of copepods as the chief source of food for several types of commercially valuable fish and whales is well known (Hardy, 1924; Wimpenny, 1929; Hjort and Ruud, 1929; Savage, 1931; and Campbell, 1934). Many other fish and various invertebrates depend upon copepods for food directly or indirectly. Since copepods derive their nourishment from more primitive organisms, their rôle may be regarded as that of "middle-man" in the main food-chain of the sea (Clarke, 1934*a*). The nutrition of copepods is, however, a matter about which very little is definitely known. The experiments described in this paper were therefore undertaken in an attempt to discover the precise organisms upon which copepods feed. This information is desired not only in relation to their growth but also in relation to their distribution (Clarke, 1934*b*). Furthermore, the development of a suitable method of culturing copepods in the laboratory has long been desired in order that the effects of temperature, light, and other important factors upon the growth and behavior of the animals could be investigated under carefully controlled conditions. To accomplish this a knowledge of the food of copepods is obviously a primary prerequisite.

PREVIOUS INVESTIGATIONS

Previous investigations of the food of copepods have been largely inconclusive, as already pointed out (Clarke, 1934*a*; cf. also Yonge, 1931). In general the observations may be divided into those which are claimed to indicate that copepods live on diatoms and those which appear to indicate that other organisms are the chief source of food. Examinations of the alimentary canals of copepods carried out by Dakin (1908), Esterley (1916), Marshall (1924), and Lebour (1922) revealed that a major part of the recognizable material was composed of diatom fragments. Crawshay (1915) kept *Calanus finmarchicus* alive in a pure culture of the diatom, *Nitzschia closterium*, for several weeks. Campbell (1934) believes that diatoms are the chief source of food for *Calanus tonsus* in the Strait of Georgia. She states, however, " It does not seem so essential that a large supply of food be available while the copepod is in the adult stage. During the fifth copepodid stage there is probably sufficient food stored as oil to tide the organism over the egg-producing period. . . . The very young stages of the copepod probably do not depend as much as the older stages upon the diatoms for food. Minute Protozoa and bacteria doubtless form the chief constituents of their diet."

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[1] Contribution No. 66.

Marshall, Nicholls, and Orr (1934) report that in Loch Striven during 1933 the periods of diatom increases coincided with the three main spawning periods of *Calanus finmarchicus*. They assert: " It is, of course, impossible to state what are the actual requirements of a *Calanus*, but the younger stages being less mobile, will require a richer supply than the later stages and adults. The critical time is therefore the period from egg to early copepodite, and the presence or absence of diatoms in the water then, means the success or failure of a brood." In addition they found that " The remaining constituents of the microplankton (chiefly minute flagellates) although at times numerous show no relation to breeding periods or survival." Thus Marshall, Nicholls, and Orr feel that diatoms are essential to the nauplii whereas Campbell believes that diatoms are an important source of food only during the late copepodid stages.

In contrast to these observations are those of Bigelow (1926), Johnstone (1911), and Fish (1925), in which an unmistakable decrease in the amount of zoöplankton was found to take place whenever diatoms were abundant. Fish's explanation is that " the common species [of diatoms] having these swarming periods do not form the food of the zoöplankton so far as I have been able to determine," and that " During the maxima of the large diatoms the smaller members of this group which are eaten by pelagic animals disappear, causing a scarcity in the food supply."

In at least two previous cases copepods have been kept in the laboratory on food other than diatoms. Murphy (1923) reared *Oithona nana* in small Stender dishes in each of which a small piece of fresh kelp was placed. Although a fair growth of *Navicula* and small Protozoa occurred, the copepods were observed to eat the kelp in large amounts. Bond (1933), using a culture of the green flagellate, *Platymonas*, as a source of food, reported that *Calanus finmarchicus* lived for over two months, *Euchaeta* sp. for over five weeks, and *Tigriopus fulvus* for several months including many generations. Bacteria were not excluded from the culture media in either of these cases nor in the experiments of Crawshay cited above. Since copepods appear to be well equipped with enzymes (Bond, 1934), their diet should not be limited from lack of powers of digestion.

MATERIAL AND METHODS

The following copepods obtainable within a three hours run of Woods Hole were used in the present investigation: *Centropages typicus* and *hamatus, Labidocera aestiva, Acartia tonsa*, and *Calanus finmarchicus*. Only about 100 specimens in several liters of water were brought in at one time and the catch was kept cold in the boat's ice box until the laboratory was reached. Immediately upon arrival the copepods were transferred by means of a large-mouthed pipette to the containers to be used for culturing. The containers were placed in one of two constant temperature tanks or in a large refrigerator. The tanks were maintained at different temperatures (kept constant to 0.1° C.) by means of two Kelvinator cooling units operated by Hiergesell thermoregulators and relays.

The sea water used in the containers was taken from the laboratory tap except where otherwise specified. In certain experiments the water was changed by pouring away all but a little of the original culture medium, leaving the copepods in the bottom corner of the dish and then replenishing with fresh sea water. The effect of stirring and aëration was investigated by using beakers equipped with plungers activated by the Plymouth siphon device (Harvey, 1928) or by employing Erlenmeyer flasks through which compressed air from a tap filter pump was slowly bubbled.

The materials which were tested as possible sources of food for the copepods included, first, the organisms already present in the sea water. In a few experiments the water was centrifuged and the material thrown down added to the culture dishes. Material taken in the harbor with a diatom net was introduced into the containers in other cases. In a large number of the experiments "persistent"[2] cultures of diatoms and green flagellates grown in the laboratory were used. With the kind assistance of Professor H. H. Gran a culture of *Nitzschia closterium* was started from specimens obtained in the vicinity of Woods Hole. The cells grew chiefly on the bottom and sides of the vessel and the copepods provided with this culture did not survive. Since one supposes that filter-feeders such as copepods (Cannon, 1928) can consume material in suspension only, this culture and others of the encrusting type were abandoned as a food source. Entirely different was the culture of *Nitzschia closterium* kindly sent me by Dr. E. J. Allen from the Laboratory of the Marine Biological Association at Plymouth. In this case the diatoms grew almost entirely in suspension and the cells themselves were quite dissimilar from the Woods Hole form, being smaller and almost straight. The following green flagellates were also found to remain in suspension in pure culture and were tried as sources of food: *Carteria mediterranea*, procured from the Pflanzenphysiologisches Institute der Deutschen Universität at Prague; *Chlamydomonas* sp., obtained from tide pools on Black Rock at the entrance of New Bedford Harbor;[3] and a mixed culture of *Dunaliella marina* and *D. salina*, kindly sent me by Dr. R. M. Bond.

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[2] By a "persistent" culture is meant one which contains only one species of alga but is not free from bacteria and Protozoa (cf. Allen and Nelson, 1910).

TABLE I

The survival of *Calanus* in relation to the presence of diatoms and green flagellates. At 15° C., small crystallizing dishes were used each containing 2 copepods in 35 cc. of filtered harbor water and 5 cc. of the food culture. At 12° C., Erlenmeyer flasks were used each containing 5 copepods in 275 cc. of filtered harbor water and 25 cc. of the food culture.

Food material added	Copepods alive after the following no. of days:					Total number of molts
	0	5	11	19	25	
	Temperature: 15° C.					
Nitzschia	10	9	4	2	2	3
Dunaliella	10	9	7	4	3	3
Carteria	10	10	6	1	0	1
Chlamydomonas	10	10	5	1	1	3
No food added	10	9	3	0	0	0
	Temperature: 12° C.					
Nitzschia	10	10	8	8	7	10
Dunaliella	10	10	5	3	1	1
Carteria	10	8	7	7	6	4
No food added	9	8	3	2	2	2

The diatoms and flagellates were all cultured by the following method recommended to me by Professor H. H. Gran. Sea water from the laboratory tap was filtered, heated to 70° C., and allowed to cool. To each liter was added 10 cc. of the following nutrient solution: 0.1 per cent KNO_3 and 0.01 per cent Na_2HPO_4 in distilled water. Growth was improved by adding also to each liter of sea water 5–10 cc. of a soil extract made as follows: To 1 kg. of rich, dark garden soil add 1 liter of distilled water. Autoclave at a pressure of 15 lbs. for

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[3] I am indebted to Professor W. R. Taylor for assistance in locating and identifying this organism.

30 minutes. Decant and filter. (Sterilize, if extract is not used immediately.) The diatom cultures were placed in a north window, the flagellate cultures in a south window but shielded from direct sunlight. When heavily inoculated, a good growth was obtained in large Pyrex Erlenmeyer flasks after about 10 days.

EXPERIMENTS USING DIATOMS AND GREEN FLAGELLATES

Preliminary experiments revealed that the copepods would die off rapidly if the temperature was allowed to rise above 20° C. or if the animals were overcrowded. When the culture water was not changed, an allowance of 20 cc. of water per copepod appeared to be adequate provided that the animals did not tend to cluster in one part of the culture dish. When provided with various of the food sources already mentioned (*Dunaliella*, *Chlamydomonas*, and *Carteria* were found to be equally efficacious), survival in the cases of *Centropages, Acartia*, and *Labidocera* was improved, but the majority of the animals did not live for moré than about two weeks. In most of the cases in which the flagellates were added to the water, green material could be seen in the intestines of the copepods and many excretory casts were found on the bottom of the containers. Molted shells were observed only rarely. Stirring, aëration, and changing the culture water—thus avoiding the accumulation of metabolites—seemed to have no ameliorating effect. However, since we have not yet developed a completely successful method of keeping copepods alive in the laboratory, it is impossible to decide in many cases what the precise cause of the death of the animals was.

In the case of *Calanus finmarchicus* only a slight improvement in survival was found to result from the addition of green flagellates and diatoms to the culture water (Table I). The copepods, the majority of which were in copepodid Stages IV or V when taken, were examined every few days and any dead animals or molted shells were removed from the containers. Two specimens lived for 25 days in water to which nothing had been added. There appears to be no consistent difference in the effects of the several organisms tried as sources of food, but it is clear that more animals survived and more molted at 12° C. than at 15° C.

In a more elaborate experiment with *Calanus* (Table II) it was similarly found that the addition of *Dunaliella* did not enable a significantly larger proportion of the copepods to survive. However, this treatment resulted in a definitely increased number of molted shells. No significant difference appears to exist between the survival in sea water taken from the laboratory tap and in that taken directly from the harbor. Of the two temperatures, the lower permitted a larger proportion of the copepods to survive but about the same total number of molted shells was observed. At the higher temperature growth would presumably be more rapid and hence a larger number of molted shells would be expected. However, since the period of ecdysis is known to be a critical one (cf. Hagmeier, 1930), it is possible that an increased rate of molting resulted in more deaths. Fewer animals would therefore be left to produce shells. A high mortality in connection with ecdysis might account in part for the better survival of the copepods at the lower temperature.

Table II

The survival of *Calanus* in relation to the type of sea water with and without the addition of food material. Erlenmeyer flasks were used each containing 5 animals in 300 cc. water including, in the cases indicated, 25 cc. of the *Dunaliella* culture.

Source and treatment of water	Copepods alive after the following no. of days					Total no. of molts
	0	5	11	17	25	
	Temperature 15° C.					
Lab. supply, untreated, plus *Dunaliella*	20	18	13	12	11	8
Lab. supply, untreated, no food added	5	5	3	3	3	5
Lab. supply, autoclaved, plus *Dunaliella*	20	19	13	10	9	11
Harbor water, untreated, plus *Dunaliella*	20	18	15	15	15	13
Harbor water, untreated, no food added	5	5	3	3	3	2
Harbor water autoclaved, plus *Dunaliella*	15	12	11	9	5	9
Harbor water autoclaved, no food added	5	5	4	1	0	2
	Temperature 5–6° C.					
Lab. supply, untreated, plus *Dunaliella*	20	19	19	18	16	15
Lab. supply, untreated, no food added	5	5	5	5	5	0
Lab. supply, autoclaved, plus *Dunaliella*	15	13	11	10	10	7
Lab. supply, autoclaved, no food added	5	5	5	2	2	1
Harbor water, untreated, plus *Dunaliella*	20	19	19	18	18	19
Harbor water, untreated, no food added	5	5	5	5	4	1
Harbor water, autoclaved, plus *Dunaliella*	15	13	12	10	10	6
Harbor water, autoclaved, no food added	5	5	4	4	4	1

In this experiment an even larger proportion of the *Calanus* survived when no food material was added. This aroused the suspicion that the copepods were feeding on microörganisms or other material already present in the culture water. In every case, survival was better in untreated water than in autoclaved water, and for the most part more shells were molted in the former medium. Although the sterilized culture water became contaminated immediately by bacteria introduced with the living copepods, the untreated water would presumably contain a richer supply of microörganisms. If the copepods could be shown to be able to derive nourishment from these organisms, the better survival in the untreated water would be explained. The possibility existed that in all of the experiments the green flagellates and diatoms were not being assimilated at all, or only to a slight extent, and that the ameliorating effect of adding these materials was due to the bacteria or other organisms which are present in "persistent" cultures (Waksman et al., 1933) and which might be used by the copepods as a source of food.

TABLE III

The survival of *Calanus* in relation to the presence of microörganisms.

Crystallizing dishes with 2 animals in each were used for the first and third sets of tests, Erlenmeyer flasks with 5 animals in each were used for the second set. Temperature 5–6° C.

Treatment of water (Harbor water)	Original no. of copepods	Copepods alive after 1 month
Berkefeld filtered	50	22
" " plus *Nitzschia*	47	8
Untreated	50	39
Untreated	15	9
Filtered through paper	15	9
Berkefeld filtered	15	0
Berkefeld filtered plus *Nitzschia*	15	8
Berkefeld filtered	40	0†
" " plus bacteria*	36	9†

* Culture of common forms taken from Woods Hole harbor and grown on agar slants kindly supplied by Dr. C. L. Carey.

† After three weeks.

EXPERIMENTS USING MICROÖRGANISMS

To investigate the possibility that the copepods could derive nourishment from bacteria, three sets of tests were run in which the particulate matter was removed by passing the water through a Berkefeld filter (Table III). In the first set of tests less than half the *Calanus* in the Berkefeld-filtered water survived and in the other tests all succumbed. In the untreated water a definitely larger proportion of copepods was alive at the end of one month. The addition of *Nitzschia* did not improve the survival, and in the first test it was attended by a very high mortality, although the reason for this is not known. Passing the water through a paper filter evidently did not remove material necessary for the copepods, since the survival under these circumstances was no poorer than in the case of the untreated water. Finally, when bacteria were added to the Berkefeld filtered water, the mortality was not as great as it was in the control experiment without the bacteria.

Although the results of these experiments seem to support the suggestion that the copepods were depending more on minute organisms than on the larger diatoms and green flagellates for food, their empirical nature renders them far from conclusive. The complete flora of the culture dishes is not known, nor were its changes followed during the course of the experiment. Furthermore, the length of time that *Calanus* can live without any food is in doubt because even the Berkefeld-filtered water was contaminated as soon as non-sterile copepods were placed in it.

Since no method for sterilizing copepods has yet been devised to our knowledge (cf. Bond, 1933), the interference from extraneous bacteria was minimized by causing fresh culture media to flow continuously through the flasks within which the copepods were confined—a scheme resorted to previously for a similar purpose (Gellis and Clarke, 1935). A continuous flow was provided by running the culture water through glass tubes from carboys placed on a shelf through submerged cooling coils to 500 cc. Erlenmeyer flasks set in the constant temperature tank (15° C.). Each flask was fitted with a two-hole rubber stopper through which inflow and outflow tubes passed. The end of the outflow tube within the flask was covered with netting of bolting silk and its level adjusted so that 400 cc. of liquid remained in the flask. The rate of flow was kept constant by arranging the carboys as " Mariotte's Bottles " (McCarthy, 1934) and the flow was suitably regulated by stopcocks to allow the entire contents of each carboy (22 liters) to pass through the flask connected with it in 24 hours.

In the first experiment sea water from the laboratory tap was passed through Whatman No. 12 filter paper and placed directly in Carboy *A*. The water for Carboy *B*, after being filtered through paper, was passed through the Berkefeld filter to remove the particulate matter. Although small quantities of water may be rendered absolutely sterile by Berkefeld filtering, complete removal of bacteria is not to be expected when large amounts of water are required. However, the existence of a significant difference in the numbers of bacteria present in the two containers was indicated by a bacterial count (using agar plates with suitable dilutions) made 12 hours after the carboys had been filled. Carboy *A* was found to contain 680,000 bacteria/cc. and Carboy *B*, 120,000/cc.

Twenty specimens of *Calanus finmarchicus* were placed in each flask. After 20 days a total of 8 dead animals and 5 molted shells had been removed from Flask *A* (paper-filtered water). From Flask *B* (Berkefeld filtered water) 12 dead animals and no molted shells had been removed. In Flask *A* 11 copepods were still alive (one animal missing), whereas in Flask *B* only 2 copepods had survived (6 missing). It is clear, therefore, that in the Berkefeld-filtered water more animals were known to have died, many fewer were found still alive, and none had molted.

These results so strongly supported the idea that the copepods derived an important part of their nourishment from minute organisms that a second experiment using flowing water and designed to provide much greater differences in the numbers of bacteria present was immediately undertaken. To obtain a culture medium more nearly completely devoid of particulate matter, a more effective method of removing the bacteria was required. Resort was therefore made to a Zsigmondy ultra-filtration apparatus, using membrane filters of the "fine" type (average pore size about one micron to fifty millimicrons; said to remove "positively all germs and bacteria" and "the finer colloids"). Harbor water was collected from the Laboratory float and passed through Whatman No. 12 filter paper, then through the Berkefeld filter, and finally through the membrane filter. After this process, which required 6 hours or more, the water was placed in one of the three carboys used in this experiment (Carboy No. 3, Table IV). Into another of these carboys (Carboy No. 1) was put harbor water which had been passed through filter paper only and thus contained its original population of bacteria unaltered. An attempt was made to compare with this "normal" water not only water from which the bacteria had been removed but also water in which the number of bacteria was augmented. Dr. S. A. Waksman had found that if sea water was brought into the laboratory and left standing in the dark at room temperature, the number of bacteria increased enormously, reaching a maximum after 3 days. Accordingly, in the remaining carboy (Carboy No. 2) was placed harbor water which had been passed through filter paper and then allowed to stand for 3 days (or 2 days, see below).

A 24-hour supply of the 3 types of culture media was prepared every day, and just before use, cotton-filtered air was drawn through each for half an hour to insure the presence of sufficient oxygen. In this experiment two flasks were connected to each carboy; the tests were thus run in duplicate. The ends of both the inflow and the outflow of every flask were covered by netting, thus precluding the possibility of the animals escaping up the tubes. As a control, a fourth pair of flasks was set up which was not provided with flowing water. The paper-filtered harbor water placed in these remained unchanged throughout the experiment. Each of the 8 flasks contained 20 *Calanus* in 300 cc. of water. All were examined every day and any dead animals and molted shells were removed.

At the end of 14 days it was found that the copepods in the flowing, paper-filtered water (Flasks 1*A* and *B*, Table IV) had fared the best, a total of 35 specimens being still alive, and 32 shells having been molted. The next best survival occurred in the controls (Flasks 4*A* and *B*), in which the water remained unchanged, with 33 copepods alive and 20 molted shells. In the paper-filtered water which had been allowed to stand (Flasks 2*A* and *B*) 28 animals were alive and 15 molts had occurred. Survival in the membrane-filtered water (Flasks 3*A* and *B*) was decidedly inferior, only 7 animals being alive and no molts occurring. In five cases the dead and living animals totaled only 19 and in one case they totaled 21. These discrepancies may be due to errors in counting when the copepods were first placed in the flasks. But 4 animals were missing from Flask 2*B* and 5 animals from Flask 3*B*, and in the previous experiment 6 animals from Flask *B* were unaccounted for. Since in the last two cases the culture water was supposedly deficient in food, the possibility suggests itself that the missing animals were devoured by the others (cf. Lebour, 1922). On one occasion a copepod

TABLE IV

The survival of *Calanus* in flowing water in relation to the abundance of bacteria. Twenty copepods per flask. Temperature: 12° C. Results at the end of 14 days. Roman numerals refer to the copepodid stages of *Calanus*.

Flask	Culture water	Total dead	Total molts	Alive
1*A*	Paper filtered	1 Cent.,* 1 Lab.	10 V, 5 IV	2♂, 9♀, 6 V = 17
1*B*		1 Cent., 2 V	12 V, 5 IV	0♂, 12♀, 6 V = 18
2*A*	Paper filtered, allowed to stand	1♂, 1 V	8 V, 1 IV	0♂, 8♀, 8 V, 1 IV = 17
2*B*		1 Cent., 3 V, 1 IV	3 V, 3 IV	0♂, 4♀, 6 V, 1 IV = 11
3*A*	Membrane filtered	8 V, 4 IV	0	0♂, 2♀, 5 V = 7
3*B*		1♀, 13 V, 1 IV	0	0 = 0
4*A*	Paper filtered, water unchanged	1 Cent., 1 V	5 V, 4 IV	0♂, 8♀, 9 V = 17
4*B*		3 V	7 V, 4 IV	2♂, 9♀, 5 V = 16

* Four *Centropages* and one *Labidocera* were found to have been introduced by mistake. They were all among the first to die.

which had been alive on the previous day was found in a half demolished condition.

Through the kind assistance of Dr. C. L. Carey it was possible to make bacterial counts of the culture media at three times during the course of this experiment. On the first day after the beginning of the experiment the following numbers of bacteria were found:

Flask 1*A*	580,000 bacteria/cc.
Flask 2*A*	130,000 "
Flask 3*A*	6,000 "
Carboy 3	140 "
Flask 4*A*	210,000 "

This count showed that a very pronounced difference existed in the sizes of the bacterial populations in Flasks 1*A* and 3*A*, but contrary to expectation there were fewer bacteria in Flask 2*A* than in Flask 1*A*. A more elaborate count was therefore made on the fifth day:

Carboy	Days after collecting	Bacteria per cc.	Flask	Bacteria
1	0	6,000	1*A*	88,000
2	4	9,000	2*A*	28,000
2*a*	3	1,000		
2*b*	2	45,000		
2*c*	1	14,000		
3	1	8,000	3*A*	220,000

The maximum number of bacteria in the standing carboys was evidently reached after 2 days instead of after 3 days. The procedure of the experiment was altered accordingly, and beginning on the seventh day, Carboy No. 2 was allowed to stand only 2 days. Furthermore, the populations in Flasks 1*A* and 2*A* are very much smaller than they were on the first day, and the population in Flask 3*A* has become enormously increased. In all three flasks the numbers of bacteria were much greater than in the corresponding carboys which were supplying them. The explanation for this is not known. Evidently the flora is subject to violent and rapid fluctuations, and in any future attempt to study the precise relationships between bacteria and copepods it will be necessary to follow these changes much more closely than was possible in the present case.

The extremely large number of bacteria found on this occasion in Flask 3*A*, which was supposed to be devoid of particulate matter, aroused the suspicion that the filtering process was faulty. The following counts were therefore made on the seventh day.

Carboy 3 immediately after filtering	9	bacteria/cc.
" " " " aërating	7	"
" " 17 hours after starting flow (1 cc. direct)	88	"
" " " " " starting flow (10–1 dil.)	530	"
Flask 3*A* " " " starting flow (10–1 dil.)	890	"

These results indicate that the use of membrane filters is as effective in removing the bacteria as could be expected for such large volumes of water. In addition, it seems probable that the large population apparently present in Flask 3*A* on the fifth day was exceptional, and that taking the whole period of the experiment, the copepods in this flask were provided with a decidedly smaller number of bacteria than those in the other flasks.

There is no question but that the copepods in Flasks 1*A* and *B* were thriving throughout the course of the experiment and that those in Flasks 3*A* and *B* were suffering adverse conditions. Little can be concluded regarding the copepods in Flasks 2*A* and *B* because of the uncertainty as to the numbers of bacteria present. It may be significant, however, that all but two of the molts which occurred in these flasks took place after the seventh day—when the period for which the water was allowed to stand was changed from 3 days to 2 days. Good survival and a relatively large number of molts were found in the control flasks (4*A* and *B*) although 40 animals would have been expected to have consumed all the food material present in a very few days. The bacteria originally in the culture water undoubtedly could multiply faster than the copepods would eat them provided that sufficient nutrient substances for the bacteria were present. Although in plain sea water the nutrients would be largely exhausted in three or four days, it is possible that the material excreted by the copepods served as a supplementary source of nourishment for the bacteria and permitted a sizable flora to maintain itself for the fourteen days of the experiment.

If the foregoing interpretation is accepted, the experiment appears definitely to point to the dependence of the *Calanus* on bacteria or other microörganisms. But a possible alternative explanation of the whole experiment should be considered, namely, that none of the copepods were feeding to any significant extent and that the differences in the three tests were due to other factors than the nutritional. Several investigators have suggested that at certain times under natural conditions copepods may live for long periods of time with a greatly reduced diet (cf. Campbell, 1934; Marshall, 1924). In the present experiment the elaborate filtering process used for Flasks 3*A* and *B* might conceivably have rendered the culture water harmful in some respect, and the period of standing employed in the case of the water for Flasks 2*A* and *B* might have allowed lethal bacteria to multiply unduly. The slight improvement of Flasks 1*A* and *B* over Flasks 4*A* and *B* could be explained on the basis of a better supply of oxygen and removal of metabolites in the flasks with flowing water. However, the difference between these two sets of flasks is very slight in respect to survival, but in number of molts, the former set is superior by 50 per cent; and in the other cases longer survival is correlated with a greater amount of molting. Although it is possible that stored oil could be entirely depended upon for the production of new shells, the growth entailed in the process would seem to presuppose the taking in of a considerable quantity of nutriment.

Everything considered, the interpretation of the experiment on the nutritional basis seems the more probable one. According to this, the copepods were living and growing on bacteria or other food material small enough to pass through the pores of the paper filter, and when this particulate matter was removed, the animals failed to grow and died off rapidly.

DISCUSSION

Since the time of Lohmann it has been known that the nannoplankton constitutes a large *potential* source of food in the sea, but little has been done to show whether this is actually used by the zoöplankton (cf. Bond, 1933). The foregoing experiments are by no means conclusive, but if, as they indicate, copepods in nature feed to any significant extent upon material as minute as bacteria, a knowledge of the precise organisms and relationships involved will be necessary for an understanding of this part of the food-cycle of the sea. Besides the bacteria, of which there are many types in the sea, other kinds of particulate matter —both living and non-living—should be scrutinized as possible food sources. A fresh-water entomostracan, *Daphnia*, has been shown to be able to derive a certain amount of nourishment from material present in colloidal form (Gellis and Clarke, 1935). Colloidal material undoubtedly exists in sea water, but little or nothing is known of its abundance or availability. Dr. J. B. Lackey, who was conducting a survey of the Protista of Woods Hole Harbor at the time that these experiments on *Calanus* were in progress, has very kindly informed me that of the organisms, other than bacteria, present in the harbor water which would pass in any significant number through Whatman No. 12 filter paper, the following were the most numerous: the spermatozoa of fish and of invertebrates, the gametes or swarm spores of algae, flagellate Protozoa, and unidentified algal cells 2–5 μ in diameter. Of these the flagellate Protozoa would constitute the largest bulk of food material.

The majority of the diatoms—at least the larger ones—was presumably removed by the filter paper; but diatoms and all other larger organisms may be important as a food source when broken into fragments during the course of their disintegration after death. Furthermore, after the bodies of dead plants and animals have passed finally into solution, their substance serves as nutriment for countless bacteria. Larger organisms may thus indirectly furnish nourishment for the copepods. The excretions of living organisms possibly also support a useful population of bacteria. There is some evidence that part of the material synthesized by diatoms dissolves out of the living cells into the surrounding water (Pütter, 1924; Gran, 1927; Marshall and Orr, 1930; and Krogh, Lange, and Smith, 1930). Waksman et al. (1933) found that in the Gulf of Maine many more bacteria existed in association with the zoöplankton and the phytoplankton than in the free water. They state:

"A decided parallelism was observed between the abundance of diatoms in the sea and abundance of bacteria. . . . These results seem to point definitely to the fact that the development of phytoplankton in the sea is accompanied closely by bacterial development. The bacteria feed upon the excretion products of the diatoms, algae, and animal forms and probably upon these plankton forms themselves as soon as they die. . . ." The explanation of those cases in which a correlation is claimed between the abundance of copepods and of diatoms may possibly be that the copepods depend for their nourishment upon the diatoms *indirectly* through the bacteria.

SUMMARY

1. The importance of copepods in the economy of the sea and the inconclusive and contradictory nature of previous observations on their food make it desirable to investigate the nutrition of these animals in a thorough manner. For this purpose and in order to develop a satisfactory method of culturing copepods in the laboratory so that their physiology might be studied under carefully controlled conditions, feeding experiments were undertaken using *Centropages, Labidocera, Acartia,* and particularly *Calanus.*

2. Small containers without stirrers appeared to be suitable provided that over-crowding was avoided and a low temperature maintained. The materials which were tested as possible sources of food included the organisms present in the harbor water and "persistent" cultures of certain diatoms and green flagellates.

3. Although the survival of the first three copepods mentioned was prolonged by the addition of the diatoms and green flagellates, the

majority died off after about two weeks. In the case of *Calanus* only a slight improvement resulted from this treatment and a large proportion survived when no food organisms were added.

4. Further experiments revealed that the sterilization of the culture water and the removal of particulate matter from it was accompanied by a high mortality of *Calanus* and that the addition of bacteria to the water resulted in improved survival. To test the possibility that the copepods were utilizing the smaller types of microörganisms for food, the growth of bacteria, etc. was minimized by passing membrane-filtered water continuously through flasks in which certain of the copepods were confined. These copepods failed to molt and died off rapidly, whereas those in flasks through which paper-filtered water flowed remained alive and molted in large numbers.

5. These experiments therefore indicate that bacteria and other constituents of the nannoplankton may be an important food for copepods in the sea. Diatoms and other larger organisms may possibly serve as a source of nourishment indirectly through the bacteria, etc., which feed upon them and their excretions.

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BIBLIOGRAPHY

ALLEN, E. J., AND E. W. NELSON, 1910. On the Artificial Culture of Marine Plankton Organisms. *Jour. Mar. Biol. Ass.*, N.S., **8** (No. 5): 421.

BIGELOW, H. B., 1926. Plankton of the Offshore Waters of the Gulf of Maine. *Bull. U. S. Bur. Fish.*, **40**: 1.

BOND, R. M., 1933. A Contribution to the Study of the Natural Food-cycle in Aquatic Environments with Particular Consideration of Microörganisms and Dissolved Organic Matter. *Bulletin Bingham Oceanographic Collection*, **4** (Art. 4): 1.

BOND, R. M., 1934. Digestive Enzymes of the Pelagic Copepod, Calanus finmarchicus. *Biol. Bull.*, **67**: 461.

CAMPBELL, M. H., 1934. Calanus tonsus (Brady) as an Economic Factor in the Strait of Georgia. *Fifth Pacific Science Congress, Vancouver, B. C.*, A5.3., pp. 2003–2008.

CANNON, H. G., 1928. On the Feeding Mechanism of the Copepods, C. finmarchicus and Diaptomus gracilis. *Brit. Jour. Exper. Biol.*, **6**: 131.

CLARKE, G. L., 1934*a*. The Rôle of Copepods in the Economy of the Sea. *Fifth Pacific Science Congress, Vancouver, B. C.*, A5.5., pp. 2017–2021.

CLARKE, G. L., 1934*b*. Factors Affecting the Vertical Distribution of Copepods. *Ecological Monographs*, **4**: 530.

CRAWSHAY, L. R., 1915. Notes on Experiments in the Keeping of Plankton Animals under Artificial Conditions. *Jour. Mar. Biol. Ass.*, N.S., **10**: 555.

DAKIN, W. J., 1908. Notes on the Alimentary Canal and Food of the Copepods. *Intern. Rev. Hydrobiol. u. Hydrogr.*, **1**: 772.

ESTERLEY, C. O., 1916. The Feeding Habits and Food of Pelagic Copepods and the Question of Nutrition by Organic Substances in Solution in the Water. *Univ. Calif. Publ. Zool.*, **16**: 171.

FISH, CHARLES J., 1925. Seasonal Distribution of the Plankton of the Woods Hole Region. *Bull. Bur. Fish.*, **41**: 91.

GELLIS, S. S., AND G. L. CLARKE, 1935. Organic Matter in Dissolved and in Colloidal Form as Food for Daphnia magna. *Physiol. Zool.* (in press).

GRAN, H. H., 1927. The Production of Plankton in the Coastal Waters off Bergen, March–April 1922. *Rept. Norweg. Fish. and Mar. Investigation*, **3** (8).

HAGMEIER, A., 1930. Die Züchtung verschiedener wirbelloser Meerestiere. 1930. *Abderhalden: Handb. biol. Arbeit.*, Abt. 9, Teil 5, Heft 4, Lief. 326, pp. 465–598.

HARDY, A. C., 1924. The Herring in Relation to its Animate Environment. Part I. The food and feeding habits of the herring with special reference to the east coast of England. *Min. Agric. Fish., Fish. Invest.*, Ser. 2, **7**: 1.

HARVEY, H. W., 1928. Biological Chemistry and Physics of Sea Water. Cambridge University Press. Cambridge, England.

HJORT, J., AND T. T. RUUD, 1929. Whaling and Fishing in the North Atlantic. *Rapp. Proc. Verb. Cons. Inter. Explor. Mer.*, **56**: 1.

JOHNSTONE, J., 1911. Life in the Sea. Cambridge University Press. Cambridge, England.

KROGH, A., E. LANGE, AND W. SMITH, 1930. On the Organic Matter Given Off by Algae. *Biochem. Jour.*, **24**: 1666.

LEBOUR, M. V., 1922. The Food of Plankton Organisms. I. *Jour. Mar. Biol. Ass.*, **12**: 644.

MARSHALL, SHEINA, 1924. The Food of Calanus finmarchicus during 1923. *Jour. Mar. Biol. Ass.*, **13**: 473.

MARSHALL, S. M., A. G. NICHOLLS, AND A. P. ORR, 1934. On the Biology of Calanus finmarchicus. V. Seasonal distribution, size, weight, and chemical composition in Loch Striven in 1933, and their relation to the phytoplankton. *Jour. Mar. Biol. Ass.*, **19**: 793.

MARSHALL, S. M., AND A. P. ORR, 1930. A Study of the Spring Diatom Increase in Loch Striven. *Jour. Mar. Biol. Ass.*, **16**: 853.

MCCARTHY, E. L., 1934. Mariotte's Bottle. Science, **80**: 100.

MURPHY, HELEN E., 1923. Life Cycle of Oithona nana, Reared Experimentally. *Univ. Calif. Publ. Zool.*, **22**: 449.

PÜTTER, A., 1924. Der Umfang der Kohlensaurereduktion durch die Planktonalgen. *Pflügers Arch. Physiol.*, **205**: 293.

SAVAGE, R. E., 1931. The Relation between the Feeding of the Herring off the East Coast of England and the Plankton of the Surrounding Waters. *Min. of Agric. and Fish., Fish. Invest.*, Ser. 2, **12**: 4.

WAKSMAN, S. A., H. W. REUSZER, C. L. CAREY, M. HOTCHKISS, AND C. E. RENN, 1933. Studies on the Biology and Chemistry of the Gulf of Maine. 3. Bacteriological investigations of the sea water and marine bottoms. *Biol. Bull.*, **64**: 183.

WIMPENNY, R. S., 1929. Preliminary Observations on the Fat-content of the Plankton on the English Herring Grounds in the North Sea. *Min. of Agric. and Fish., Fish. Invest.*, Ser. 2, **11**: 5.

YONGE, C. M., 1931. Digestive Processes in Marine Invertebrates and Fishes. *Jour. du Conseil*, **6**: 175.

FURTHER EVIDENCE OF A DIURNAL RHYTHM IN THE MOVEMENT OF PIGMENT CELLS IN EYES OF CRUSTACEANS

JOHN H. WELSH

(From the Bermuda Biological Station for Research and the Biological Laboratories, Harvard University)

Diurnal rhythms which persist under constant environmental conditions have long interested biologists and although numerous instances of such are now known we still lack definite information regarding the internal processes which are involved in such rhythms. Among the many daily changes in activities of animals, some of which have been previously mentioned by the author (Welsh, 1930), the movements of the pigment cells in arthropod eyes would appear to be quite satisfactory for studying the physiological mechanism underlying persisting diurnal rhythms. Kiesel (1894) and Demoll (1911) were the first to observe a daily rhythm in the " glow " of the eyes of certain moths when these were kept in constant darkness. Welsh (1930) first reported a daily rhythm in the crustacean eye. This was in two species of *Macrobrachium*, fresh water shrimp, which were studied in Cuba. In these forms, when kept under constant illumination, the distal pigment cells of the eye assume the position characteristic for the dark at the time of sunset and do not return to the light position until sunrise the following morning. A daily variation in the position of the proximal pigment of the eye of *Cambarus*, when this form is kept in constant darkness, has been reported by Bennitt (1932). Various suggestions have been offered to explain the persistence of such rhythms but none of them are supported by adequate experimental data. The rich crustacean fauna of Bermuda should offer material for such studies and the purpose of the present paper is to show that persisting diurnal rhythms do exist in several Bermuda shrimp and prawn eyes and in all of the three sets of pigment cells found in the eyes of such forms.

If daily variations in the position of pigment cells in the eye occur, and if they continue under constant environmental conditions, they may be best detected by keeping animals in constant light or darkness and killing and fixing some during daylight hours and others during the night. Subsequent comparison of sections of such eyes would then show the condition of both light-adapted and dark-adapted eyes as they appear during the day or night. Such a procedure was used in a search for rhythms in eye pigment in Bermuda shrimp and prawn and the condition in the eyes of four species, representative of those studied, follows. The species to be described were selected as representing three families and at the same time including species which are active only at night and others which are most active during the daytime. They are all forms which are easily obtained in abundance and therefore satisfactory for experimental work.

In preparing material for study, animals were light-adapted or dark-adapted for several hours and then fixed in hot water, some during the day and others at night. The head bearing the eyes was then cut off and placed in 70 per cent alcohol. At least three eyes fixed under each of the four conditions were then sectioned and studied and the typical condition is shown in Plate I. The single ommatidia are scale drawings adapted from camera lucida sketches and in each ommatidium the positions of the distal, proximal, and accessory pigments are shown. For general information regarding the movement of retinal pigment one may refer to the excellent review by Parker (1932). It may be well to mention here, however, that all three sets of pigment are involved in controlling the amount of light which reaches the rhabdomes or image receptors. Typically, in the light condition during the day, the distal pigment cells rest on top of the retinular cells, the proximal pigment surrounds the rhabdomes, and the accessory or reflecting pigment is beneath the basement membrane. Fig. *B* in each series shows this condition. The normal dark condition as seen at night is shown in Fig. *C* of each series. In this condition the distal pigment cells move out along the cones, the proximal pigment migrates below the basement membrane, and the reflecting (white) pigment moves up to form a screen back of the rhabdomes. This situation permits the maximum amount of light to enter the sensitive elements.

Any variation in the position of the pigment in eyes of light-adapted animals fixed at night (Fig. *A*) or of dark-adapted animals killed during the daytime (Fig. *D*) would indicate some internal control other than that resulting from the direct stimulation of the eye by light or

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Explanation of Plate I

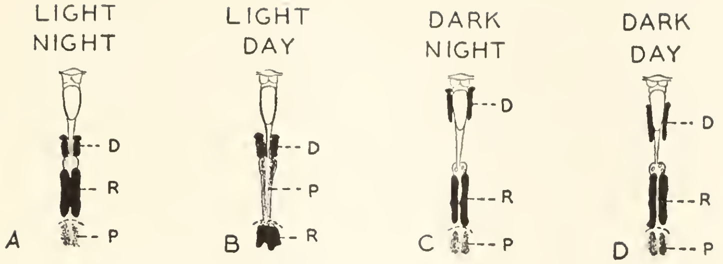
Series 1. Ommatidia of eyes of *Latreutes fucorum* (× 100) showing positions of distal, proximal, and reflecting pigment of (*A*) light-adapted animals killed at night; (*B*) light-adapted animals killed during day; (*C*) dark-adapted animals killed during night; (*D*) dark-adapted animals killed during day.

Series 2. Ommatidia of eyes of *Leander tenuicornis* as in Series 1, (× 50).

Series 3. Ommatidia of eyes of *Leander affinis* as in Series 1, (× 50).

Series 4. Ommatidia of *Penaeopsis goodei* as in Series 1, (× 50).

D = distal pigment.
P = proximal pigment.
R = reflecting pigment.


LIGHT NIGHT
LIGHT DAY
DARK NIGHT
DARK DAY
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R
P
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P
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P
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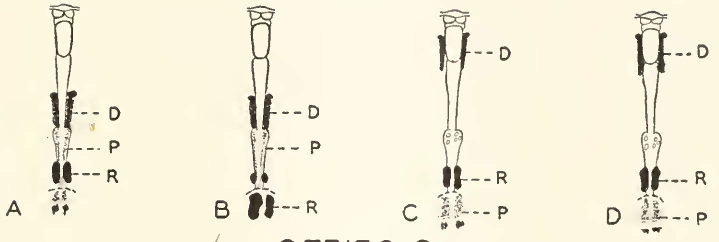
SERIES 1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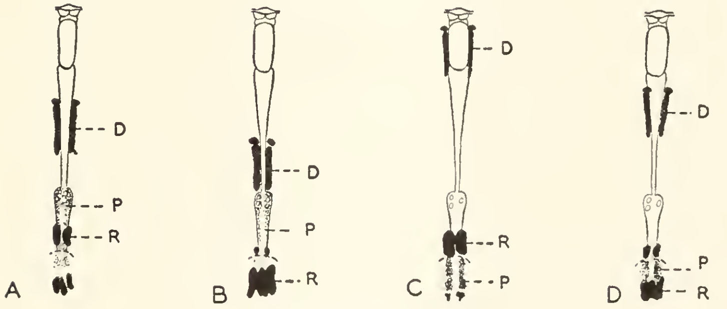
SERIES 2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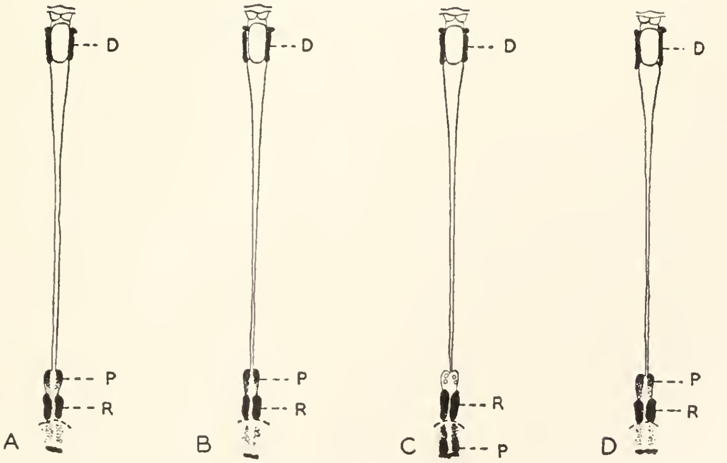
SERIES 3


D
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D

SERIES 4

Plate I

the lack of such a stimulus. In each series some such variations may be seen and these variations will be pointed out in the consideration of each separate species.

Latreutes fucorum (Fabr.) Stebbing

Family: Hippolytidae. "Gulf-weed Shrimp."

This species is found on Sargassum weed in large numbers where it is associated with the other very characteristic fauna of the gulf-weed. It is well protected by its color resemblance to the weed and its tendency to remain quiescent during the day. Its exposure to fairly brilliant illumination during the day necessitates a mechanism in the eye to care for a wide range of light intensities.

When the eyes of this form are studied, the most striking rhythm is seen in the reflecting or accessory pigment (Figs. *A–D*, Series 1). If we compare the ommatidia from "night-light" eyes with those from "day-light" eyes, it is seen that during the night, even though the animals are subjected to illumination, the reflecting pigment still remains above the basement membrane. Slight variations from normal dark and light conditions may also be seen in the positions of the distal and proximal pigment.

Leander tenuicornis (Say)

Family: Palaemonidae. "Common Gulf-weed Shrimp."

The habits and habitat of this form are like those of *Latreutes*. Similarly the most striking rhythm is in the reflecting pigment (Figs. *A–D*, Series 2). This is of some interest as *Leander* and *Latreutes* belong to different families.

Leander affinis (Milne-Edwards)

Family: Palaemonidae. "Transparent Shrimp."

This species of *Leander* differs considerably from *L. tenuicornis* in its habits. It is active chiefly during the daytime, when it is found in schools close to the shore. It is well protected by its relative transparency, which renders it difficult to see. Light and dark-adapted ommatidia from eyes fixed during the day and during the night are seen in Figs. *A–D*, Series 3. A definite rhythm in the movements of the reflecting pigment and a partial migration of the distal pigment occur daily whether the animals are kept in constant light or darkness.

Penaeopsis goodei (Smith)

Family: Penaeidae.

The habits of this species of prawn were probably observed for the first time while this study was being made. They are of sufficient interest to recount briefly. *P. goodei* is a distinctly nocturnal form found swimming at the surface in the late evening between 8:30 and 10:30 P.M. It can easily be seen by the brilliant reflection of light from the eyes when a search-light is played over the surface of the water. During the day this prawn is found buried in the sand. Specimens were obtained on two occasions when dredging for other sand-dwelling forms.

Besides a very definite daily periodicity in activity this form is also found swimming at the surface in largest numbers at the dark of the moon, so that it may be said to exhibit a lunar periodicity as well. The observations made during the course of this work have since been further substantiated by observations made by Dr. J. F. G. Wheeler, Director of the Bermuda Station.

When the eyes of *Penaeopsis* are examined (Figs. *A–D*, Series 4), it is seen that the distal pigment cells fail to show any movement. They remain in a fixed position around the distal portion of the cones. Likewise the reflecting pigment maintains the same position in the light or in the dark. The only movement which occurs is in the proximal pigment, which migrates below the basement membrane when animals are dark-adapted during the night. During the daytime, however, even though the animals are kept in the dark the proximal pigment returns to the retinular cells, which is the normal light position. The only rhythm in this form, therefore, is that of the proximal pigment, when animals are kept in constant darkness. In this respect *Penaeopsis* resembles *Cambarus*.

It is evident from this and earlier studies that diurnal rhythms involving changes in eye pigment are widespread among such crustaceans as shrimp and prawn. It now remains to be shown what underlying physiological rhythms are involved. The most recent evidence which promises to aid in an understanding of this phenomenon in crustacean eyes is that of Kleinholz (1934), who has shown that the eye-stalk hormone, first found by Perkins (1928) and Koller (1928) to affect the body chromatophores, also plays a part in the movement of the distal pigment cells. Kleinholz has demonstrated that an extract of the eye-stalks of light-adapted animals when injected into animals which have been kept in the dark causes a complete migration of the distal pigment cells to the position characteristic of the light. More recent unpublished results indicate that the eye-stalk hormone has no effect on the proximal or reflecting pigments. This is the first convincing demonstration of hormone effects on crustacean eye pigments and also furnishes additional evidence indicating that the different pigments of the eye are under different controls. Apparently it is possible to obtain by means of the eye-stalk hormone a situation opposite to that in the eyes of *Macrobrachium* (Welsh, 1930), where by keeping the animals under constant illumination the distal pigment, at night, assumes the position characteristic of the dark, while the proximal pigment remains in the light condition.

If diurnal rhythms under constant conditions occurred only in the movements of the distal pigment cells, we would seem to be approaching an explanation of the underlying process, but the fact that such rhythms also occur in the movements of the proximal and reflecting pigments only makes a complicated situation more difficult to understand.

In conclusion the author wishes to acknowledge his indebtedness to Doctors G. L. Clarke and F. A. Brown, who collected in Bermuda some of the material used in this study, to Dr. F. A. Chace, Jr., who did the identifications, and to Miss Virginia Macdonald, who assisted in the histological preparation of the material.

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LITERATURE CITED

Bennitt, R., 1932. Diurnal Rhythm in the Proximal Pigment Cells of the Crayfish Retina. *Physiol. Zool.*, **5**: 65.

Demoll, R., 1911. Über die Wanderung des Irispigments im Facettenauge. *Zool. Jahrb., Abt. allg. Zool. u. Physiol.*, **30**: 169.

Kiesel, A., 1894. Untersuchungen zur Physiologie des facettirten Auges. *Sitzungsber. Akad. Wiss., Wien.*, Abt. 3, **103**: 97.

Kleinholz, L. H., 1934. Eye-stalk Hormone and the Movement of Distal Retinal Pigment in Palaemonetes. *Proc. Nat. Acad. Sci.*, **20**: 659.

Koller, G., 1928. Versuche über die inkretorischen Vorgänge beim Garneelenfarbwechsel. *Zeitschr. vergl. Physiol.*, **8**: 601.

Parker, G. H., 1932. The Movements of the Retinal Pigment. *Ergebnisse der Biologie*, **9**: 239.

Perkins, E. B., 1928. Color Changes in Crustaceans, Especially in Palaemonetes. *Jour. Exper. Zool.*, **50**: 71.

Welsh, J. H., 1930. Diurnal Rhythm of the Distal Pigment Cells in the Eyes of Certain Crustaceans. *Proc. Nat. Acad. Sci.*, **16**: 386.

A CORRELATION BETWEEN THE FOOD EGGS OF FASCIOLARIA TULIPA AND THE APYRENE SPERMATOZOA OF PROSOBRANCH MOLLUSCS

J. WENDELL BURGER AND CHARLES STEAD THORNTON

(From the Department of Biology, Princeton University)

It has long been known in *Fasciolaria tulipa* that of at least 600–800 eggs laid in a capsule only a few develop. Those eggs that fail to develop are eaten by the normal larvæ. Glaser (1905), in a study of this "egg cannibalism" in *Fasciolaria*, has suggested that these food eggs represent a condition comparable to that found in oligopyrene sperm, such as Meves (1901) described for *Paludina*. During a study of Professor E. G. Conklin's preparations of *Fasciolaria*, we have found cytological evidence that permits an elaboration of Glaser's suggestion. Moreover, because of the more recent work of Reinke (1912, 1914) on the apyrene sperm of *Strombus*, it is possible to make a more adequate correlation between atypical eggs and spermatozoa.

The material examined consists of eggs prepared by Professor E. G. Conklin in 1903 at Beaufort, N. C. In addition to a series of untreated eggs, several series were treated with the following parthenogenetic reagents:

1 per cent NaCl for 1½ hours
2 per cent NaCl for 12 hours
4 per cent $MgCl_2$ for 10 hours
8 per cent $MgCl_2$ for 12 hours
1 drop of HCl in 300 cc. sea water.

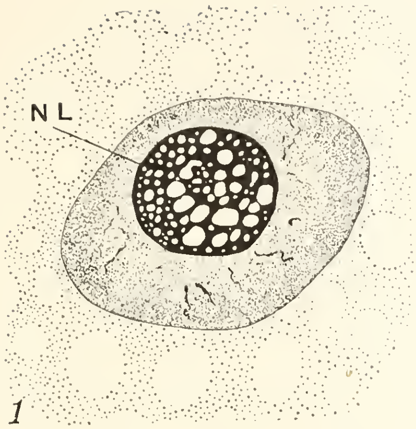
We are indebted to Professor E. G. Conklin for his kindness in putting his preparations at our disposal and for suggesting this study.

In cytological detail the food eggs of *Fasciolaria* are more comparable to apyrene sperm than to oligopyrene ones. Meves (loc. cit.) described a type of spermatocyte in *Paludina* which had abnormal maturation divisions, wherein the chromosomes were irregularly distributed to the resulting cells; further the spermatids transformed abnormally. These spermatozoa were designated "oligopyrene sperm." In 1912 Reinke, working with *Strombus,* found that the primary atypical spermatocytes did not undergo any maturation division but transformed directly into abnormal spermatozoa. Their nuclei fragment into coarse chromatin granules, which become vesiculate, fade, and disappear. This type of sperm is called "apyrene."

The food eggs of *Fasciolaria* also have no maturation divisions. When laid they have a large, faintly reticulate germinal vesicle in which there is an abnormal giant nucleolus (Fig. 1). Usually the nucleus remains in this condition until degeneration occurs. Glaser (1907) has described this degeneration as an "explosive" process, whereby the nucleus is broken into vesicles containing bits of the nucleolus. He failed to find evidences of amitosis such as Osborn (1904) described. We have been able to find stages intermediate between the germinal vesicle and the fragments observed by Glaser; for frequently the nucleolus and the nuclear membrane are distorted (Fig. 2), suggesting that this distortion will lead to fragmentation in a somewhat slower manner than that described by Glaser, although the result is the same.

In other eggs there are feeble attempts at a maturation division. In some of these eggs the fine chromatin reticulum of the germinal vesicle becomes condensed into imperfectly formed chromosomes. These chromosomes are grouped together within the nuclear membrane; the membrane itself may be distorted so that the chromosomes are somewhat separated from the nucleolus (Fig. 3). On the other hand, the chromosomes may form more perfectly and arrange themselves in the semblance of a mitotic figure, in which no spindle fibers are to be seen (Figs. 4 and 5). Here the nucleolus may or may not disappear or become separated from the chromosomes.

The attempt at division goes no farther than this. The nucleus and chromosomes undergo degeneration in much the same manner as described for the chromatin granules of the atypical spermatocytes of *Strombus*. Vacuolate chromosomal vesicles appear, with the deeply staining chromatin lining their walls. These vesicles enlarge and their contents stain less and less deeply. During this process the nucleolus may or may not fragment. In many cases the nucleolus can be seen to break into several faintly staining bodies which become scattered in the cytoplasm; but in other instances the nucleolus does not fragment, but becomes very faintly stained and is surrounded by a ring of degenerating vesicles (Fig. 6). The treatment with parthenogenetic reagents did not cause development of the eggs. The important fact is, as Osborn, Glaser, and we have found, that normally there are no maturation divisions in the food eggs of *Fasciolaria*. This fact presents a situation comparable with the apyrene sperm of *Strombus*, and other forms. That all the eggs are not arrested in the same stage presents a correlation with the spermatozoa of this species; for Hyman (1923) has described three types of atypical spermatozoa.



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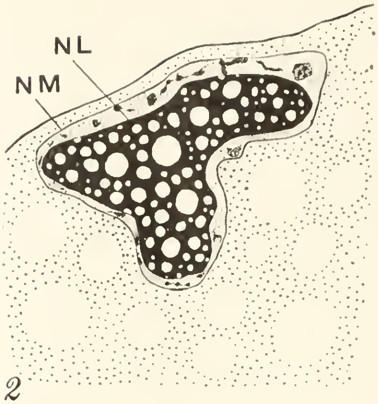

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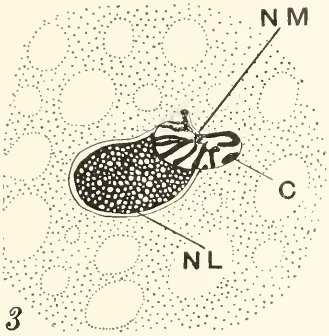

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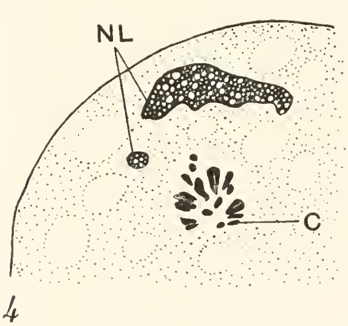

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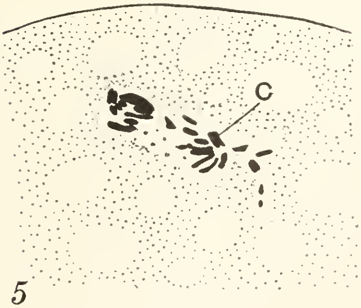

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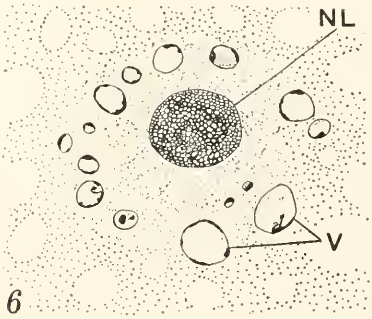

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FIG. 1. Germinal vesicle before fragmentation. Nucleolus, (*nl*) large and vacuolate. Yolk represented by blank areas.

FIG. 2. Lobulate nucleus with nucleolus extending nearly to nuclear membrane (*nm*).

FIG. 3. Chromosomes (*c*) condensed and collected at one pole of the nucleus. Nuclear membrane still intact.

FIG. 4. Polar view of the chromosomes. Nucleolus undergoing fragmentation.

FIG. 5. Chromosomes arranged in a semblance of a mitotic figure. Nucleolus has disappeared.

FIG. 6. Chromosomal vesicles (*v*) forming a ring around the nucleolus. Degenerating stage.

Bütschli (1877) described irregular cleavages in *Paludina* eggs, in which there was an early arrest of development. Similarly *Buccinum* and *Purpura* show irregular cleavages, but in these cases maturation divisions occur. Hence, it is suggested that these atypical eggs of *Paludina, Buccinum,* and *Purpura* are comparable to oligopyrene sperm, whereas in *Fasciolaria* they are comparable to apyrene sperm. In the limited material of *Buccinum* at our disposal we have found abnormally large polar bodies, and in the cleavage cells, degeneration of chromatin.

The significant fact which arises from this study is that in the gametogenesis of both sexes of prosobranch molluscs, there exists among the various species a transition from the normal condition to one that is highly atypical. The least atypical deviation from the normal spermatogenesis is the *Paludina* type (oligopyrene), where there are maturation divisions but these are irregular. In the *Strombus* type (apyrene) there is a situation which is more accentuated than that of *Paludina,* for here both maturation divisions are suppressed. A similar transition is found in oögenesis. In *Buccinum* and other forms there are oligopyrene eggs wherein the deviation from the normal is less marked than in *Fasciolaria.* In the latter the maturation divisions are suppressed so that only vestiges of the process remain.

It is interesting to note that in *Fasciolaria* Hyman (1923) found that the ratio of atypical spermatozoa to normal was 50:1, which ratio is of about the same order of magnitude as that found between the atypical and typical eggs. Hyman, however, failed to realize that there are atypical eggs in *Fasciolaria;* but he considered that the failure of most of the eggs to develop was due to the fact that the great number of atypical spermatozoa produced did not leave enough typical sperm to fertilize them.

Since this paper was submitted for publication, a paper has appeared by Woodard (1935) on the apyrene spermatozoa of *Goniobasis laqueata.* In the formation of these atypical spermatozoa an abortive attempt at maturation is made, wherein "a variable number of bivalent chromosomes are formed in the nucleus by aberrant prophase processes; these are brought upon an abortive spindle, then scattered to all parts of the cell where they undergo progressive vacuolization." This condition in *Goniobasis* is closely paralleled in *Fasciolaria* (cf. Figs. 3–6), and presents another link in the evidence for the apyrene egg.

LITERATURE CITED

Bütschli, von O., 1877. Entwicklungsgeschichtliche Beiträge. *Zeitschr. f. wiss. Zool.,* 29: 216.

Glaser, O. C., 1905. Über den Kannabalismus bei Fasciolaria tulipa (var. distans) und deren larvale Excretionsorgane. *Zeitschr. f. wiss. Zool.,* **80:** 80.

GLASER, O. C., 1907. Pathological Amitosis in the Food-Ova of Fasciolaria. *Biol. Bull.,* **13:** 1.

HYMAN, O. W., 1923. Spermic Dimorphism in Fasciolaria tulipa. *Jour. Morph.,* **37:** 307.

MEVES, F., 1901. Über die sog. wurmförmigen Samenföden von Paludina und über ihre Entwicklung. Separat-Abdruck aus Mittheilungen für den Verein Schlesw.-Hölst. Aertze." Jahrgang X, nr. 1, 1901, s. 1–11.

OSBORN, H. L., 1904. Amitosis in the Embryo of Fasciolaria. *Am. Nat.,* **38:** 869.

REINKE, E. E., 1912. A Preliminary Account of the Development of the Ayprene Spermatozoa in Strombus and of the Nurse Cells in Littorina. *Biol. Bull.,* **22:** 319.

REINKE, E. E., 1914. The Development of the Apyrene Spermatozoa of Strombus bituberculatus. Marine Biological Laboratory at Tortugas. v. 6, pp. 197–239.

WOODWARD, T. M., 1935. Spermic Dimorphism in Goniobasis laqueata (Say). *Jour. Morph.,* **57:** 1.

A BUFFERED AND LOW OXYGEN CONTENT PHYSIOLOGICAL SALT SOLUTION

MERLE RANDALL AND THOMAS C. DOODY

(*From the Chemical Laboratory of the University of California*)

A physiological salt mixture in which *Trichonympha campanula*, a protozoan symbiont of *Zootermopsis angusticollis*, could be kept alive was worked out by Cleveland (1925*a*). Protozoa are known to be extremely sensitive to osmotic pressure. The cell walls disrupt either inward or outward according as the osmotic pressure of the medium is larger or smaller than that of the fluid within the cell. The solution which Cleveland found most suitable was a salt mixture of approximate pH 7.2 and with an osmotic pressure equivalent to that of an approximately 0.40 per cent sodium chloride solution. This mixture was suitable for the Protozoa of many termite species, but the intestinal Protozoa of *Reticulitermes hesperus* could be kept alive in it for only a short length of time. Since the completion of our work, Trager (1934) has reported a salt mixture most suitable for the Protozoa of *Zootermopsis angusticollis* as having an osmotic pressure equivalent to that of a 0.3 to 0.4 per cent sodium chloride solution, and a pH range of 6.8 to 7.2.

Oxygen gas is used under pressure for the defaunation of termites (Cleveland, 1925*b*, *c*, *d*, 1928; Andrew, 1930). The oxygen thus forced into solution in the intestinal fluid is evidently an agent toxic to the Protozoa. This led to the suspicion that some of the intestinal Protozoa of *Reticulitermes hesperus* were unusually sensitive to dissolved oxygen and were affected by the small amount dissolved in Cleveland's solution. Trager (1934) has also later shown that some of the Protozoa of *Zootermopsis angusticollis* are very sensitive to oxygen. These grow best where air is excluded by vaseline seals or where the culture tubes connected by paraffined stoppers and glass tubing to other tubes contain alkaline pyrogallol thereby creating an atmosphere of low oxygen content. The amount of oxygen dissolved from the air at atmospheric pressure by water (Winkler, 1889) is small, being 8.8 ml. of oxygen per 1,000 ml. of water at 5.2° C., 7.1 at 14.8° C. and 5.8 at 24.8° C.

A desirable physiological salt mixture for studying the life history of these organisms should have the following properties: (*a*) The salt solution should have a low oxygen content; (*b*) excess power of absorbing oxygen must be maintained to keep the amount of oxygen in the solution low during use; (*c*) it must have the correct pH—i.e., be nearly neutral; (*d*) the osmotic pressure of the solution must be correct; (*e*) the solution must not contain constituents toxic to the Protozoa.

Manganese hydroxide has the power of removing oxygen quite completely from solutions. In the reaction manganous hydroxide is oxidized to manganic hydroxide by the dissolved oxygen.

$$Mn(OH)_2(\text{solid}) + \tfrac{1}{4}O_2(\text{dissolved}) + \tfrac{1}{2}H_2O(\text{solvent}) = Mn(OH)_3(\text{solid}). \quad (1)$$

Both hydroxides are quite insoluble. This reaction is made the basis of standard methods of quantitative determinations of dissolved oxygen in water analysis (Winkler, 1888).

Although there is considerable confusion in the literature regarding the solubility of manganous hydroxide, the value taken is 0.00043 gram manganous hydroxide, or 4.8×10^{-6} mols per liter, determined by Almkvist (1918) for the solubility in distilled water at 22° C., without correction for dissolved carbon dioxide. These conditions are approximately those found under the conditions of use of a physiological salt solution. Considering manganous hydroxide as a strong base the pH of the saturated solution would be about 9. This value is indicated by the electrometric titration of manganous chloride with sodium hydroxide (Britton, 1925; Atkins, 1923), in which the precipitation of manganous hydroxide occurs between pH values of 8.6 and 9.2. It is evident by the application of the mass law to the reaction,

$$Mn(OH)_2(s) = Mn^{++} + 2OH^- \quad (2)$$

that an increase in the concentration of the manganous ion will decrease the concentration of the hydroxide ion by precipitating more solid manganous hydroxide. Computations will show that the pH of the solution is about 7.1 when the manganous concentration is 0.025 mols per liter and about 7.0 when it is 0.050 mols per liter.

Although work on the pH of the termite intestine (Randall and Doody, 1934) showed a pH of about 6.8 in the hindgut, the success of Cleveland with a solution of pH = 7.2 led to the use of a saturated solution of manganous hydroxide (with excess of the insoluble manganous hydroxide), having a concentration of manganous ion of 0.025 mols per liter (estimated pH = 7.1). The excess of the light flocculent manganous hydroxide furnishes a plentiful reservoir of oxygen remover to take care of any oxygen absorbed during the use of the solution and acts as a buffer to retain the proper pH. There is no indication from the literature or from preliminary experiments with the manganous hydroxide that it should have any toxic effect on the intestinal Protozoa of the termite.

Mixing the Deoxygenating Solution

Boil more than one liter of distilled water vigorously for ten or fifteen minutes to remove most of the dissolved oxygen. It is best to use hot water in preparing the solution so that as little oxygen as possible will be redissolved. Dissolve 3.94 grams of manganous chloride ($MnCl_2$) in 25 ml. of the boiled water. To the remainder of one liter of the water (975 ml.) add 0.5 gram of sodium hydroxide. Pour this into a narrow necked liter flask which can be tightly stoppered. Add the manganous chloride solution, stopper tightly, and mix well by shaking. When cool, the deoxygenating solution is ready for use except that an adjustment for its osmotic pressure is required. The procedure as prescribed makes 0.56 gram per liter of insoluble manganous hydroxide, of which only 0.0000048 gram is dissolved. This provides a plentiful excess of the insoluble hydroxide. Always shake the mixture well before using, so there will be particles of the insoluble deoxygenating hydroxide in the solution which is to be used. As the dissolved manganous hydroxide is used it is replaced by more of the insoluble hydroxide going into solution.

The insoluble manganous hydroxide, $Mn(OH)_2$, is light pink in color. The manganic hydroxide, $Mn(OH)_3$, which is formed by reaction with the dissolved oxygen, is also insoluble; but its color is dark brown. This dark brown color appears during the preparation of the deoxygenating solution, due to the removal of the slight amount of oxygen dissolved in the water of the solution. This does no harm as there still remains an ample excess of the undissolved deoxygenating agent, manganous hydroxide. The solution should be kept in a tightly stoppered flask, as it deteriorates rapidly due to the absorption of oxygen from the air.

Adjustment for Osmotic Pressure

The osmotic pressure of the above saturated and buffered solution of manganous hydroxide is too low as was evident from the disruption of the cells of the Protozoa in preliminary experiments.

The salt mixture used by Cleveland was roughly equivalent to a 0.40 per cent solution of sodium chloride, which we found to give approximately the right osmotic pressure. Therefore 0.740 gram of sodium chloride should be added to each liter of the manganous hydroxide solution. A finer adjustment of the osmotic pressure is made from observations on the effect upon the Protozoa when the solution is used as a medium.

A more suitable medium for these Protozoa could probably be made by adjusting the osmotic pressure with one of the sugars which can be produced by the decomposition of cellulose. The hexoses ($C_6H_{12}O_6$) such as glucose, galactose, and mannose, can be produced from cellulose, and would probably show no ill effect in the intestine of an animal which lives on cellulose. The approximate adjustment of the osmotic pressure of the manganous hydroxide medium can be made by the addition of 6.67 grams of glucose, galactose, or mannose, or any mixture of them, to each liter of the medium.

Action on the Protozoa of Reticulitermes Hesperus

The method used by Dr. O. L. Williams of the Sub-committee on Biology, Termite Investigations Committee, to examine the Protozoa of *Reticulitermes hesperus* was as follows: The intestinal contents of a termite were mixed with about 0.5 ml. of the fluid medium containing manganous hydroxide (osmotic pressure adjustment made with sodium chloride). The depression in a hollow-ground slide was then filled with this dilution, and a cover-slip sealed in place with vaseline or paraffin.

Preparations made as above showed active Protozoa for periods of twenty-four hours or longer. Preparations made in the same manner with Cleveland's solution in place of the above described manganous-containing solution showed active Protozoa only during periods of less than two hours.

Mr. J. E. Gulberg, who has used the manganous solution in taking micro-motion pictures of the Protozoa *R. hesperus,* where the intense illumination required is a serious factor in limiting the survival of the Protozoa, found that it was possible to keep the organisms alive over a period of about two hours. This was accomplished without other special precautions to exclude oxygen from the preparations which were being photographed. Under the same severe conditions, with the usual physiological salt solutions, the organisms could not be kept alive more than a few minutes.

These results show considerable advantage for the preparations of the Protozoa from *Reticulitermes hesperus* in which the amount of dissolved oxygen is reduced. The manganous hydroxide mixture is itself a diluent of very low oxygen content. It also supplies a reserve oxygen-removing power which absorbs oxygen from the material which was diluted and maintains the mixture to be observed at very low oxygen content. The use of this manganous hydroxide mixture adds no complications to present technique and allows the production of essentially oxygen-free cultures more easily than with any of the methods in common use. The manganous hydroxide mixture as described above gives a buffered solution of adjustable pH and osmotic pressure which appears non-toxic to Protozoa.

Slight changes in the pH of the solution, the composition of the added salts, and the adjustment of the osmotic pressure can be made to suit other organisms requiring physiological salt solutions of low oxygen content.

We wish to make acknowledgment to the Termite Investigations Committee under whose auspices this work was done. We also take this opportunity to thank Mr. J. E. Gullberg and Dr. O. L. Williams for their coöperation.

BIBLIOGRAPHY

ALMKVIST, G., 1918. *Zeitschr. anorg. allgem. Chem.*, **103**: 240.
ANDREW, B. J., 1930. *Univ. Calif. Publ. Zool.*, **33**: 449.
ATKINS, W. R. C., 1923. *Trans. Faraday Soc.*, **18**: 310.
BRITTON, H. T. S., 1925. *Jour. Chem. Soc.*, **127**: 2110.
CLEVELAND, L. R., 1925*a*. *Biol. Bull.*, **48**: 282.
CLEVELAND, L. R., 1925*b*. *Biol. Bull.*, **48**: 295.
CLEVELAND, L. R., 1925*c*. *Biol. Bull.*, **48**: 309.
CLEVELAND, L. R., 1925*d*. *Biol. Bull.*, **48**: 455.
CLEVELAND, L. R., 1928. *Biol. Bull.*, **54**: 231.
RANDALL, M., AND T. C. DOODY, 1934. Chapter 8 of "Termites and Termite Control," University of California Press, Berkeley, California.
TRAGER, WILLIAM, 1934. *Biol. Bull.*, **66**: 182.
WINKLER, L. W., 1888. *Ber. deutsch. chem. Gesellschaft*, **21**: 2843.
WINKLER, L. W., 1889. *Ber. deutsch. chem. Gesellschaft*, **22**: 1764.

THE CHROMOSOMES OF HERMAPHRODITES

I. Lepas anatifera L.[1]

EMIL WITSCHI

(From the Zoölogical Laboratory, State University of Iowa, and the Marine Biological Laboratory, Woods Hole, Mass.)

Our information relative to chromosomes of hermaphrodite animals is still very scanty, which is deplorable in view of the fact that a general theory of the sex chromosome ought to be based on hermaphrodite as well as gonochorist forms. The only cases exhaustively studied are those of the nematode *Angiostomum* (*Rhabditis*) *nigrovenosum* (Boveri, 1911; Schleip, 1911) and the coccid *Icerya purchasi* (Hughes-Schrader, 1927, 1930). Both are atypical insofar as the " hermaphrodites " begin development as females, morphologically as well as with respect to chromosome numbers. When, later on, testicular islands and spermatozoa are formed, they arise only in consequence of a localized process of sex reversal which is accompanied by chromosomal regulations. In the case of *Angiostomum* one X chromosome becomes inactive and subsequently is omitted, while in *Icerya* the diploid set of chromosomes is reduced to the haploid. In either case, the chromosomal condition of the male is established. Indications of the regulation become visible at the first spermatocyte stage or even earlier. No somatic cells of male constitution are formed. Hermaphrodism, in these cases, is visibly secondary to the gonochorist condition.

There is good reason to assume that a vast majority of hermaphrodites are of a different type and have not been derived from gonochoristic ancestors. Thanks to data accumulated in papers by Boveri (1890), Stevens (1903, 1904, 1910), Buchner (1910), Elpatiewsky (1910) and Bordas (1912, 1914), we obtain a fairly complete picture of the chromosome cycle in several species of *Sagitta*. The diploid number of 18 chromosomes is found in somatic cells as well as in spermatogonia and ovogonia. Mature eggs and spermatozoa all contain 9. This situation corresponds to that which generally is considered to be characteristic for hermaphrodite and monoecious higher plants. In *Datura stramonium*, Belling and Blakeslee show that the diploid number is 24 and the haploid 12. That the chromosome sets of

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[1] This investigation was supported by grants from the Committee for Research in Problems of Sex of the National Research Council.

pollen grains and eggs are qualitatively equivalent is demonstrated by genetical evidence. Hence there exists a well established type of hermaphrodism with sex differentiation that is not followed by chromosomal rearrangements in either sex. We designate this condition as primary hermaphrodism, since it appears to be the prototype from which the different forms of gonochorism in Metazoa and in higher plants have arisen.

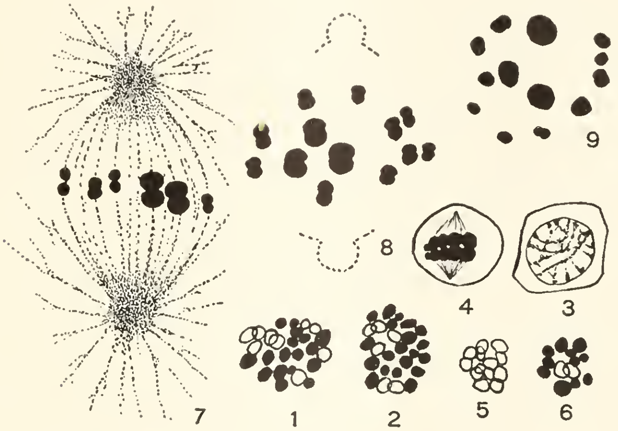
In the hermaphrodite annelid *Ophryotrocha puerilis*, A. and K. E. Schreiner (1906) as well as Huth (1933) have demonstrated the presence of 8 chromosomes in somatic cells and in dividing gonia. In the maturation divisions of spermatocytes and ovocytes appear four chromatic units, and from Huth's careful morphological analysis one can conclude that all ripe gametes receive equivalent haploid chromosome sets.

Perrot (1934), in his recent study on *Limnaea stagnalis*, gives 18 as the haploid number in both male and female maturing germ cells. The necessity of further critical studies in gastropod chromosome cycles is, however, emphasized by an earlier paper of the same author (1930).

In respect to the problem of the evolution of gonochorism the order of the Cirripedia would seem to be of particular interest. A large number of genera are straight hermaphrodites. Others consist of large hermaphrodites and smaller "complementary" males, while a few little studied species have large female and small male forms. The present paper deals with the goose barnacle, *Lepas anatifera* L., a common hermaphrodite. The material was collected at the Marine Biological Stations of Roscoff, France and of Woods Hole, Massachusetts.

In the intestinal walls of adult specimens relatively frequent mitoses are found which lend themselves favorably to chromosome counts due to the large size of the cells. Prophase and metaphase chromosomes appear like beads of different size, the largest measuring about eight times the volume of the smallest ones. In the equatorial plate these chromosomes are crowded together, though in favorable cases one can easily enough establish 26 as the diploid somatic chromosome number (Figs. 1 and 2).

Spermatogenesis is characterized by the extreme smallness of the cells. The successive stages of maturation of primary spermatocytes were studied with utmost care. Slides were stained with hematoxylin after Ehrlich, Delafield and Heidenhain as well as by the Feulgen method. The chromosomes unravel and form very fine threads during the conjugation phase (Fig. 3). Heteropycnotic elements distinctly are absent. Due to the small size of the spindle, the chromosomes of the first meiotic metaphase are so closely packed that they can be individually seen and counted in a small fraction only of the innumerable dividing spermatocytes that one finds in testes during the reproductive season (Figs. 4–6). If seen in profile (Fig. 4), the chromosomes appear as paired beads. As they segregate and move toward the spindle poles they soon become rearranged, some moving faster, others slower. The minute size of the spindle simply does not permit the advance of undisturbed chromosomal plates during anaphase. No uneven distribution of the chromosomes is indicated and no chromatin elements are eliminated. The author was not able to count chromosomes in the second meiotic division, though it was ascertained that no chromosome elimination occurred here, either.



FIGS. 1–9. *Chromosomes of Lepas anatifera L.* 1, 2, dividing intestinal cells. 3, spermatocyte in conjugation phase. 4–6, spermatocytes in first maturation division. 7, profile view of first meiotic spindle in the egg. 8, chromosomes in a first meiotic spindle which stands obliquely in the section. 9, polar view of the chromosome plate of the first meiotic spindle in the egg. All figures × 2000.

Lepas, like many other crustaceans, brings periodically large numbers of eggs to simultaneous maturity. In one specimen the ovary contained many thousands of eggs, all at the stage of metaphase of the first maturation division. The spindle which lies close to the surface of the oval egg has more than three times the linear dimensions of the corresponding spermatocyte figure (compare Figs. 7 and 4). The chromosomes are well separated since they are relatively smaller (barely twice the diameter of spermatocyte chromosomes; Figs. 9 and 6). The haploid number of 13 was established with greatest ease in more than two hundred individual counts. Three large chromosomes generally occupy the center of the plate while ten smaller ones usually take their place along its periphery. The relative size differences are the same in ovocytes and spermatocytes. The fact that the individual chromosomes are at least four times as voluminous in female as in male germ cells of identical stage suggests that the visible chromatin represents much more than the aggregate volume of the genes.[2] Seen in profile the metaphasic chromosomes appear as pairs composed of two equal elements (Figs. 7 and 8).

Weismann and Ischikawa (1888) had seen " four double-chromatin-elements " in the maturation spindle of the egg, while Groom (1894) reports that " the number of chromatic elements varies; it appears to be commonly four or five, but, in some cases was, as far as I could make out, as many as ten or twelve." To prove the incorrectness of these counts we refer to the photomicrograph Fig. 10. The true number can be ascertained without difficulty even in properly stained whole mounts.

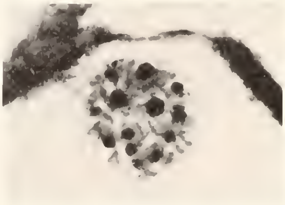


FIG. 10. Photomicrograph of the 13 chromosomes in the first maturation spindle in the egg of *Lepas*. × 1500.

No heteromorphic sex chromosomes have been found in somatic or in germinal cells of either sex. Nor have we observed any indication of chromosomal regulation of either the *Angiostomum*- or the *Icerya*-type. Even though this study does not include some of the important phases of the chromosome cycle (especially that of fertilization), the evidence obtained renders it more than probable that the goose barnacle is a primary hermaphrodite in the sense defined above. Delage (1884), G. Smith (1906), Meisenheimer (1921), and others have defended the opinion that hermaphrodism in *Cirripedia* is secondary, the order having descended from gonochoristic, free-swimming ancestors. The evidence of comparative morphology has, however, been interpreted also in the opposite sense. The chromosomal situation is distinctly in favor of Darwin's (1851, 1854) theory of primitive hermaphrodism and secondary development of "complementary" males and of complete gonochorism.

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[2] In *Ophryotrocha*, according to Huth (1933), the chromosomes of the polar spindle are of same size as those of the first spermatocyte, even though the spindles show a similar size ratio as in *Lepas*.

BIBLIOGRAPHY

Bordas, M., 1912. Contribution à l'étude de la Spermatogénèse dans la Sagitta bipunctata. *La Cellule,* **28**: 165.

Bordas, M., 1914. Doctrinas actuales sobre la reduccion numérica de los chromosomas y su aplicacion á la espermatogénesis de la Sagitta bipunctata Quoy et Gaim. *Mem. d. l. Real. Soc. Expan.,* **10**: 1.

Boveri, Th., 1890. Zellen-Studien. Über das Verhalten der chromatischen Kernsubstanz bei der Bildung der Richtungskörper und bei der Befruchtung. *Jena. Zeitschr. f. Naturwiss.,* **24**: 314.

Boveri, Th., 1911. Über das Verhalten der Geschlechtschromosomen bei Hermaphroditismus. Beobachtungen an Rhabditis nigrovenosa. *Wuerzburg Verh. physik. Ges. (N.F.),* **41**: 83.

Buchner, P., 1910. Die Schicksale des Keimplasmas der Sagitten in Reifung, Befruchtung, Keimbahn, Ovogenese und Spermatogenese. *Festschr. f. R. Hertwig., Jena,* **1**: 233.

Darwin, C., 1851–54. A Monograph of the Sub-class Cirripedia. The Lepadidae, London 1851; The Balanidae etc., London 1854.

Delage, Y., 1884. Évolution de la sacculine. *Arch. Zool. expér. et gén., 2 sér.,* **2**: 417.

Elpatiewsky, W., 1910. Die Entwicklungsgeschichte der Genitalprodukte bei Sagitta. I. Die Entwicklung der Eier. *Biol. Zs. Moskva,* **1**: 333.

Groom, T. T., 1894. V. On the Early Development of Cirripedia. *Phil. Trans. Roy. Soc. London, Ser. B,* **185**: 119.

Hughes-Schrader, S., 1927. Origin and Differentiation of the Male and Female Germ Cells in the Hermaphrodite of Icerya purchasi (Coccidae). *Zeitschr. f. Zellforsch. u. mikr. Anat.,* **6**: 509.

Hughes-Schrader, S., 1930. The Cytology of Several Species of Iceryine Coccids, with Special Reference to Parthenogenesis and Haploidy. *Jour. Morph.,* **50**: 475.

Huth, W., 1933. Zur Cytologie der Ophryotrochen. *Zeitschr. f. Zellforsch. u. mikr. Anat.,* **20**: 309.

Meisenheimer, J., 1921. Geschlecht und Geschlechter im Tierreiche. Bd. I. Jena.

Perrot, J. L., 1930. Chromosomes et Hétérochromosomes chez les Gastéropodes pulmonés. *Rev. Suisse Zool.,* **37**: 397.

Perrot, J. L., 1934. A propos du nombre des chromosomes dans les deux lignées germinales du Gastéropode hermaphrodite Limnaea stagnalis (var. rhodani). *Rev. Suisse Zool.,* **41**: 693.

Schleip, W., 1911. Das Verhalten des Chromatins bei Angiostomum (Rhabdonema) nigrovenosum. *Arch. Zellforsch.,* **7**: 87.

Schreiner, A., and K.E., 1906. Die Reifung der Geschlechtszellen von Ophryotrocha puerilis Clprd.-Mecz. *Anat. Anz.,* **29**: 465.

Smith, G., 1906. Rhizocephala. Fauna Neapel, Berlin, **29**: 1.

Stevens, N. M., 1903. On the Ovogenesis and Spermatogenesis of Sagitta bipunctata. *Zool. Jahrb., Abt. f. Anat.,* **48**: 227.

Stevens, N. M., 1904. Further Studies on the Ovogenesis of Sagitta. *Zool. Jahrb., Abt. f. Anat.,* **21**: 243.

Stevens, N. M., 1910. Further Studies on Reproduction in Sagitta. *Jour. Morph.,* **21**: 279.

Weismann and Ischikawa, 1888. Weitere Untersuchungen zum Zahlengesetz der Richtungskoerper. *Zool. Jahrb. Abt. f. Anat.,* **3**: 575.

CENTRIFUGING THE EGGS OF ILYANASSA IN REVERSE

T. H. MORGAN

(From the William G. Kerckhoff Laboratories of the Biological Sciences, California Institute of Technology, Pasadena, California)

INTRODUCTION

A study of the factors involved in the formation of the so-called yolk-lobe or antipolar-lobe in the snail *Ilyanassa* carried out in the summer of 1932 led to certain provisional conclusions concerning the nature of the lobe. Most of the experimental work was carried out by centrifuging the eggs at different stages previous to and during the formation of the lobe. The main problem was to discover whether the lobe is only an expression of changes taking place elsewhere in the egg—changes that are directly or indirectly correlated with mitosis—or whether the lobe is, in some sense, a predetermined region of the egg, and its formation merely incidental to the other phases in the mitotic or other phenomena. The view presented in the following pages is that neither of these interpretations is strictly true, because while the lobe region may be regarded as present in the ripe eggs, its delineation is not strictly preformed, and to a limited extent it possesses a certain autonomy of its own, and is independent of the mitotic phenomena as such. It is even independent of the materials contained inside that part of the egg. It should be recalled in this connection that the first time the lobe appears (Plate I, 1) it is scarcely recognizable, and can not be said to be constricted from the rest of the egg. The egg merely becomes pear-shaped with a pointed or rounded region barely outlined at the antipole. On the other hand, when the second lobe appears the lobe is a conspicuous part of the egg (Plate I, 2–7), but it never becomes much constricted at the base from the rest of the egg. The third lobe (Plate I, 9–13) at first resembles the second very closely, but later it is completely constricted from the egg when the first division is completed. The fourth lobe (Plate I, 17–18) is little more than the constriction near the middle of one (the larger) of the dividing cells. Subsequently one of the two cells is pinched off from the yolk, the other remaining attached to the yolk by the constricted region (Plate I, 18–19). This cell soon fuses with the yolk (Plate I, 20), giving the typical four-cell stage.

Between lobe-forming stages, the lobe may be said to return into the egg. This is true at least after the first and second lobe formation, while the third lobe fuses with one of the first two blastomeres (Plate I, 15–16) which does not become spherical. The fourth lobe remains as a part of one of the four blastomeres, fusing with it into one large oval cell (Plate I, 17–20).

It may also be recalled that when the first lobe appears the first polar body is coming off. The mitotic figure at the pole is very small at this time, although before passing to the pole it was quite extensive. On the other hand, the second lobe is large. At the time of its beginning the second polar body is being extruded. The mitotic figure of the second polar body is no larger than that of the first. This lobe continues to grow larger, during which time the egg-pronucleus is de-

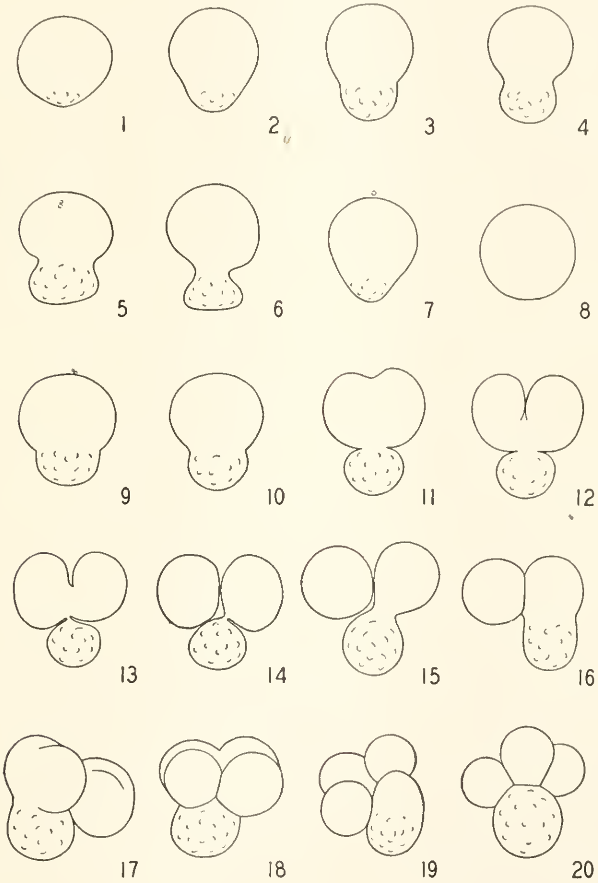

Plate I

veloping, and the sperm-pronucleus is approaching to fuse with it. When the third lobe develops, there is a very large mitotic figure in the upper hemisphere. The lobe is fully delimited at the time when the cleavage begins to cut into the upper hemisphere.

It is obvious that the extent, and in some degree the size of the lobe, is not intimately bound up with a particular stage of the mitotic figure, and that it is a more or less independent development in the antipolar hemisphere of the egg.

By means of centrifuging the eggs upside down ("in reverse"), I attempted to find out whether the formation of the lobe is due to materials contained within it, and whether its location is dependent on the location of the mitotic spindle. It has been surmised for other forms—leeches, molluscs and polychaetous annelids whose eggs also develop lobes somewhat like those of *Ilyanassa*—that the lobe contains preformed material essential for certain later embryonic structures. If this is correct, then it might seem to follow that the formation of the lobe itself is connected with the presence of these materials in the antipolar hemispheres. The experiments that I have carried out show that this is not the case, since the lobe will still develop when its materials have been removed and carried into the polar hemisphere. It may, of course, be true that "organ-forming substances" may be present in some of these eggs with lobes as has been claimed, but even if this is true it does not seem probable in the light of the experiments that the mechanics of lobe-formation has anything to do with the presence of such materials. This statement does not mean that there are not preformed structures in the antipolar hemisphere that are involved in lobe-formation. I am inclined to believe that in the surface layers of this region, that can not be removed by centrifuging, the key to the situation may be found. In support of this view is the fact that when the upper hemisphere is pinched off from the rest of the egg by centrifuging in raffinose, the cleavage of this half is into two and four equal cells. No lobe appears. The other half, the lower hemisphere, or the "bottom" will develop a lobe, even when the female and male nucleus are absent.

There are three separate but interdependent topics discussed in this and two following papers: the behavior of the egg when its interior materials are partly or wholly reversed; the cleavage of the "tops" and of the "bottoms" after their separation; the rhythmic formation of the lobe, either in the presence of the egg-chromatin alone (the sperm-nucleus having been removed), or in the total absence of all chromatin.

EGGS CENTRIFUGED IN REVERSE

Introduction

The chromatin in eggs that have just been laid and centrifuged before the first polar body has been extruded is, in the inverted egg, carried along one side, or even into the antipolar hemisphere. The sperm-pronucleus is also carried along with the protoplasm and can sometimes be found in the protoplasmic zone. Occasionally I have seen a polar body given off from the clear zone, but since the polar bodies have not often been seen in these eggs, when alive, it may be that the chromosomes of the polar spindle often divide, but do not separate into two nuclei and remain in the egg. There are some indications that a large number of the chromosomes are present when the segmentation spindle forms later at the side or in the antipolar hemisphere, but the chromosomes are so small and clumped that I have not been able to count them.

An important consideration is whether any protoplasmic readjustments take place in the inverted or in the partly inverted eggs after removal from the centrifuge. In general the whole of the protoplasm with its contained chromosomes has been carried along one side of the egg, and in completely inverted eggs it is in this way carried to the antipole. The oil before centrifuging is contained largely in the protoplasmic substance of the polar hemisphere, giving it an opaque or brownish appearance by transmitted light, or white in direct light. In well centrifuged eggs the oil leaves the protoplasm and collects as a segment at the centripetal pole. The protoplasm that lies beneath is left as a clear zone or band which stains a deep blue in haematoxylin preparations. The impression one gets when the centrifuged eggs are examined is that in some of the inverted eggs the protoplasm and oil have been carried completely to the centripetal end of the egg, while in others it has not yet been carried so far, but lies at the side. This impression is erroneous, as shown by continuing the centrifuging. It is then found that the conditions have not changed, but the same proportion of eggs still have the protoplasm at one side. In fact, the shift takes place in the first two or three minutes. The explanation is that only some of the eggs are completely inverted at the start, while many of them lie obliquely. An examination of the "inverted" eggs (in which the lobe is present) in the gelatin after it has been congealed confirms this conclusion. For example: eggs that had been "inverted" in the gelatin which was then solidified by cooling were observed in a dish of ice-water under the microscope. These eggs had a large second polar lobe, which made it possible to determine their orientation in the tube. Before centrifuging, 28 eggs were completely reversed, 59 were oblique, and 2 were not reversed at all. After centrifuging 31 were recorded as completely reversed, 2 not at all reversed, and the rest were oblique to the centrifugal force (some of these lay on their sides, Plate II, 2). This means that, as the eggs sink in the gelatin before it is frozen, some of them are completely inverted, while others lie obliquely and are centrifuged in this position. In the latter, the protoplasm slides along one side of the egg and spreads out there. The situation can be more accurately described by saying that the whole mass of yolk moves along one side, viz., towards the centrifugal pole, forcing the lighter protoplasm along the opposite side, i.e., toward the centripetal pole. When it is remembered that, unless an egg is perfectly oriented with its pole outwards, this sort of shifting is expected to take place, there can be no question but this represents the actual situation when the centrifugal force is applied. Whether in a perfectly reversed egg the yolk will be driven through the center of the egg and the protoplasm around the sides (as a ring), or whether the two materials literally pass through each other on the way to their respective poles, can not be positively asserted, but if the latter happens I have seen no evidence of it. Certainly such a method of rearrangement in the egg of *Ilyanassa* must be very exceptional.

In other kinds of eggs, where the protoplasm and yolk are more generally mixed, it is believed that the yolk granules pass individually through the protoplasm to reach the centrifugal pole and the oil droplets pass in the opposite direction. This may be, of course, what happens in such eggs, but there are indications in some of them at least, especially when the segmentation spindle is present, that the protoplasmic portion tends to remain more or less intact, and is shifted as a whole, while the yolk passes along one side toward the centrifugal pole. However this may be for other eggs, in the molluscan egg where the two regions are rather sharply defined, the rearrangement takes place by the shifting of polar and antipolar materials as wholes around and not through each other. In the frog's egg also, where the upper and lower hemispheres are well delimited, there is evidence that when the egg is not completely inverted the regions shift around each other as wholes in response to gravity.

This consideration has some interest in connection with the possible shifting back of the materials that are incompletely inverted so that the protoplasm returns to the polar end of the egg, as Conklin has described for *Crepidula*, especially in cases where the polar bodies are given off at the pole and after the protoplasm and the polar spindle have been carried away from this region. It is to be expected that different eggs may behave differently in this respect, but in *Ilyanassa* at least there is no evidence that a return movement takes place except to a minor degree. The protoplasm may concentrate around its new center after the eggs are removed from the machine, and may be supposed, if the centrifuging is not complete, to concentrate at times at one side of or even in the polar region; but the evidence from *Ilyanassa* gives little support for the view that there are any striking readjustments of this kind. When eggs with much yolk concentrated at the antipole are centrifuged in mass in the bottom of the tube, most of them will lie, at first, obliquely to the centrifugal force, and when they are not free (due to mutual pressure) to orient with the heavier end outward, the rearrangement of the contents must be like that in *Ilyanassa,* i.e., along the side. As I have said, if the centrifuging is only partial it may happen sometimes, when the greater mass of the cytoplasm remains at or near the pole, that it concentrates again near the pole. But this is very different from the implication that there is some attracting pole " force " that brings this about. The viscosity of the interior protoplasm and that of the inner layer of the surface of the egg may account for whatever rearrangement takes place.

A few statements additional to those given in my previous paper may be made with respect to the congealed gelatin technique. By using an inner tube a little larger and longer than the ones used before, a longer time is allowed for the sinking eggs to become inverted, and a higher percentage of the eggs are reversed. The outer large glass centrifuge tube should be completely filled with cracked ice, and the inner tube imbedded in this ice. Some of the ice will remain unmelted for about five minutes, especially if the brass tube of the centrifuge and the outer glass tube are cooled beforehand. When a longer time is needed, the centrifuge can be stopped, the small tube containing the eggs taken out, and a new supply of ice added. Since the eggs are in a solid medium they will not change their orientation in the interval. It is important that the smaller tube be completely filled with gelatin after the eggs are in it, so that when the forefinger is placed over it, and the tube turned over, no air bubbles are left; for if present they rise through the gelatin (then liquid) to the top, when the tube is turned over, and when the centrifuging begins they may be forced back to the centripetal end and disarrange the eggs. A little sea water is left with the eggs at the bottom of the tube when the liquid gelatin is added above it to fill the tube to the top. The eggs will not sink quickly out of this water into the gelatin unless the inverted tube is violently shaken downwards. This starts the eggs in the gelatin, and also breaks up clumps of eggs. Once started they will slowly sink in the gelatin if it is prevented from freezing by holding the other hand around the tube at this time. After a couple of minutes the eggs will be distributed along the whole length of the tube. Should the gelatin melt in the tube while on the centrifuge, the eggs may become oval, but this does not happen as long as the gelatin remains congealed. Also, in removing the centrifuged eggs from the small tube it is important not to deform them by drawing out the gelatin too rapidly before it is liquid again. This is avoided, after warming the tube in the hands, by holding the tube obliquely with the open end under warmed sea water in a flat dish, and by sending small bubbles of air upward from a pipette into the gelatin to replace it. The eggs slowly pass with the gelatin into the sea water. They must then be gently agitated to remove the gelatin and to mix it with the sea water, which is then withdrawn and replaced with new sea water.

The nuclei and chromatin of the early stages are difficult to differentiate. After Bouin's solution the haematoxylin preparations are not so good as after picrosulphuric acid, since in the former the yolk holds the stain. The most successful preparations were stained in Conklin's formula for Delafield's haematoxylin, viz., by diluting it six times with water and adding one drop of picrosulphuric acid to each 10 cc. of diluted stain. After ten minutes in the stain the eggs are washed for half a minute or less in acid-alcohol diluted to one-fourth with 70 per cent alcohol, then run up through the alcohols, etc. to balsam.

The Experiments

Repeating the experiments of the previous year, many sets of eggs were centrifuged in reverse. Preparations were made of some of these to detect the location of the egg-chromatin, the sperm-pronucleus, and the polar bodies. In eggs newly laid, the chromatin was always found in the protoplasmic zone. Since at this time there are no orienting points, it is not possible to state how many of the eggs were completely reversed. In some cases the polar bodies were seen to come off later at the side of the clear area, but it is uncertain whether or not these are eggs in which the protoplasm has been only a little displaced, and the evidence is insufficient to show that the polar bodies may be extruded at the side when the protoplasmic zone has been carried as far as and into the antipolar hemisphere. Since polar bodies were seen in relatively few cases, it follows that they are not always given off whether the reversal is complete or only partial. The yolk-lobe appeared both in those partially and in those completely reversed—the latter containing all or only part of the oil at the antipode. Some of the records of these eggs are as follows:

Newly laid eggs were centrifuged at 2,500 r.p.m. for 10 minutes in reverse (new ice added after 5 minutes). In the control the first polar body came off 10 minutes later. The second polar body was seen coming off the side of the clear zone in some of the centrifuged eggs (Plate II, 1). When the second lobe appeared it was evident that the protoplasm in many eggs lay at one side of the egg (Plate II, 2).

Only a few sets of eggs were centrifuged in reverse before the first polar spindle had come to the surface, since these stages were sufficiently examined in the two previous summers' work. In the absence of the polar bodies or polar spindles at the surface there are no points by which to orient the true antipolar hemisphere. In some cases, as reported in 1933, polar bodies came off from some of the eggs, that were only partially reversed, in the polar hemisphere, but whether exactly at the pole or near the pole in the polar hemisphere could not be definitely stated. In such cases the second lobe evidently came off at the antipole. In eggs completely reversed the chromosomes are carried into the antipolar hemisphere, as determined by the location of the second lobe. There were no cases found in which polar bodies were present in that hemisphere. The lobe developed as in the other cases. The new experiments now to be described begin with stages when the first polar spindle was at the surface, and the polar body about to be extruded.

These experiments with eggs, in reverse, at the time when the first polar body or the second polar body was just coming off, were made in order to see whether the polar spindle remains sticking at the pole when the protoplasm is driven to the side or to the antipole, and also to see, if this happens, whether the second lobe forms at the antipole. The results are not, on the whole, satisfactory, because in the first place the spindle was carried away in some cases with the protoplasm; in other cases it remained at the pole; and because, in the second place, the first polar body when free is sometimes lost and then does not serve as a marker, or becomes very difficult to detect in eggs that are reversed. In the eggs that were not reversed, hence have the pole inwards, the polar body remains attached as a rule, and stands out conspicuously from the surface of the egg. On the other hand, when the egg is reversed, the polar bodies appear often to be lost, or become difficult to detect if they lie at the outer end of the reversed egg, or at the sides of eggs partly reversed.

A set of newly laid eggs was centrifuged at 2,500 r.p.m. in reverse for 5 minutes, at the time when the first polar body was just out. The second lobe appeared 19 minutes later, both in the control and in the centrifuged eggs. The second polar spindle was then at the surface just below the polar body. Centrifuged eggs killed at once showed that the first polar body had remained at the pole. In eggs not reversed, Plate II, 3, it was above the polar protoplasm; in eggs completely reversed it lay, when found, opposite the oil (Plate II, 4); in others partly reversed (Plate II, 6) it lay at the side. In all these except that shown in Plate II, 5, the second metaphase plate was found near the first polar body.

Three other sets were centrifuged when the first polar body was coming off. The spindle was displaced in some of the eggs and carried with the protoplasm and oil even to the antipole. The results were the same as in the last case.

Eggs were centrifuged for 5 minutes when the second lobe had just appeared. They were killed as soon as removed from the machine. One partly reversed egg is shown in Plate II, 7. This egg had lain on its side. The first polar body is at the pole (possibly this is the second polar body): the protoplasm is on one side extending into the lobe; the egg-chromosomes are in the protoplasm above the equator, and the sperm-nucleus at the equator.

Another set of eggs was also centrifuged for 5 minutes, just as the second lobe was beginning. Some were killed and stained 13 minutes later (Plate II, 8–10). The first polar body was present at different points of the surface with respect to the stratification; the second spindle in metaphase lay just below it. Here the second spindle had not been shifted when the protoplasm was carried to the side or even to the antipole. In both cases the lobe developed later at the antipole. The protoplasm had not moved back to the region where the second spindle lay when the eggs were removed from the machine.

Eggs were centrifuged for 5 minutes in reverse after the first polar body had been extruded, just as the second lobe was appearing. Nineteen minutes later the lobe was fully out. The oil and protoplasm were either in it, or at the side in most eggs. When the lobe contained the oil, it was later constricted off as a rule. When protoplasm and oil were at the side, and the eggs began to round up, the lobe was not constricted off and returned to the egg. The third lobe appeared in these eggs, even when it contained most or all of the oil field. Later still, 14 eggs divided into 2 cells, mostly those in which the oil was at the side. Twenty-one eggs did not divide, 11 of which remained spherical, and 10 developed lobes (6 containing yolk and 4 oil). It is probable that the spindle had remained at the pole in these eggs, but I am unable to prove this. If so, it may not have been in position to induce cleavage because protoplasm was lacking, or because the sperm-nucleus may have been so far removed from the egg-chromatin that no cleavage spindle was formed.

Eggs were centrifuged for 5 minutes in reverse when the second lobe was coming out. It was still present when the eggs were removed from the centrifuge. In those which were actually reversed the oil constricted off later and remained outside, while in those not reversed or partially reversed it remained as part of the egg. When the third lobe appeared 13 eggs divided at one side (Plate II, 11); 7 with oil in the lobe did not divide; 4 with yolk in the lobe did not divide, and 19 remained spherical.

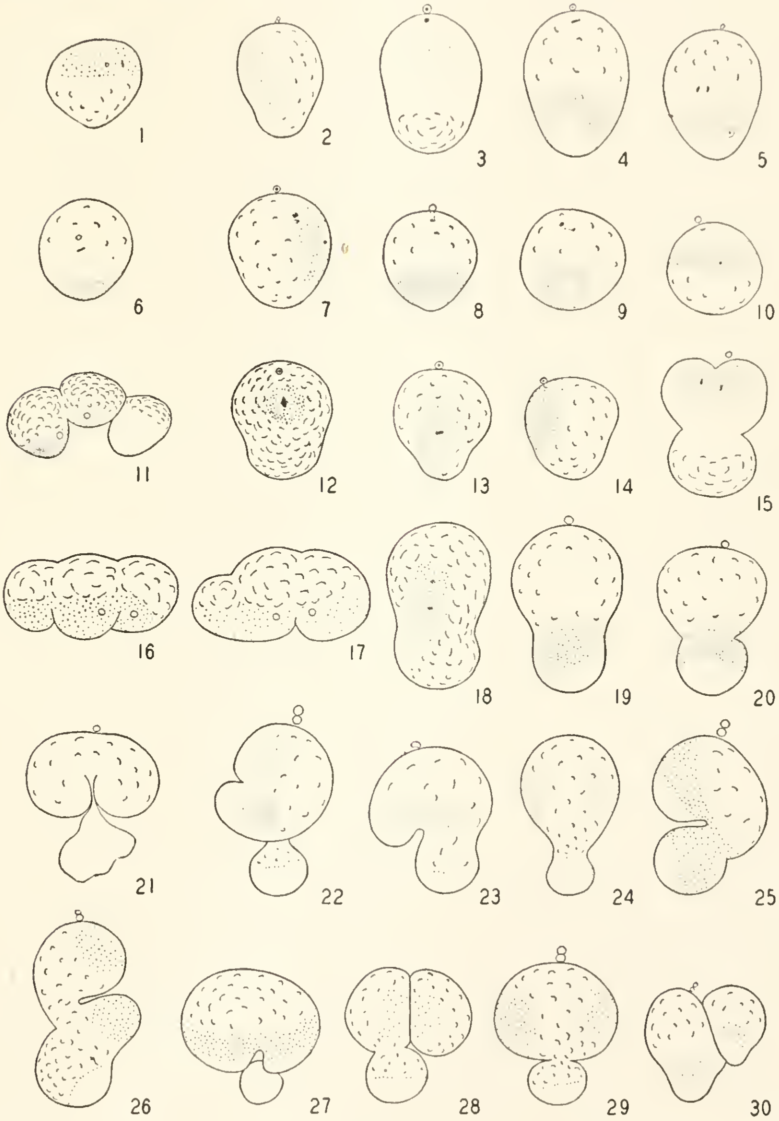
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Plate II

Eggs were centrifuged in reverse when the second lobe was halfway out. It was still present in almost all of the eggs when taken from the centrifuge. The protoplasm was in almost all of the eggs at the side; in one it was in the lobe. The lobe went back in all eggs, and reappeared later. Three eggs are represented at this time in Plate II, 12–14. In these the polar body is present at or near the true pole—the lobe is at the opposite side, the protoplasm and oil are at the side, the chromosomes are present in the middle of the protoplasm.

Eggs were centrifuged as the second lobe was just coming out. The lobe was well out 13 minutes later, and was still well developed 10 minutes later. Except in those eggs with all the oil in the lobe, the lobe went back, and about an hour later the third lobe appeared. This set was killed and drawn when the cleavage was coming on (Plate II, 15). In 15, in an egg not reversed, the division began at the pole; in Plate II, 16, 17, at one side; in the others the nucleus had divided (telophase, Plate II, 18) but the protoplasm had not constricted. The lobe is conspicuous in all of these.

Eggs with large third lobes were centrifuged for 5 minutes. The controls had begun to divide 10 minutes later. At this time in four of the centrifuged eggs (Plate II, 19, 20) the oil is in the end of the lobe, with a clear protoplasmic band beneath. The yolk is in the polar hemisphere. The controls were in two cells, 10 minutes later, but the reversed eggs did not show indications of division until two hours after removal from the centrifuge. One hour later the lobe, when it contained the oil, constricted off from the rest of the egg, and the eggs began to cleave at or near the point at which the oil (now constricted off) is present, as seen in Plate II, 21. It is into this region that the mitotic figure has been driven. In most of the eggs the constriction cut through 20 minutes later, dividing the egg into equal or nearly equal parts. Later the eggs divided irregularly. It will be noted that neither the protoplasm nor the yolk moved back to the pole in these eggs—in fact, the cleavage was about due when the eggs were centrifuged.

A few experiments were carried out in reverse when the eggs were starting to divide in order to drive the mitotic figure into the antipolar hemisphere. The point of interest here is to see if the cleavage begins at the pole, or at the new location of the spindle. Four reversed eggs are shown in Plate II, 22–25. In 25, part of the oil is in the lobe and part in the antipolar end of what would have been one of the two blastomeres. In both parts there is a clear zone next to this, containing no doubt the chromosomes. The yolk is at the pole near the two polar bodies. In 23 the conditions are similar. In 22 the oil is in the lobe, but the clear area is at the side of the bilobed region. In 24 the oil is in the lobe, and below it there is some of the clear protoplasm. There are two clear zones on the sides of this egg. Evidently most of the protoplasm of the dividing egg, already separated into two regions, has been driven along the opposite sides of the egg. The individual differences in these cases are due in part to the stage of the egg at the time of centrifuging, and in part to the angle which the partially reversed egg made with the centrifugal force. It is evident, nevertheless, that the lobe still retains its shape, more or less, when the yolk is driven out, and some of the oil and protoplasm into it, even after the egg is removed from the semi-solid jelly. Ten minutes later the condition of four eggs is shown in Plate II, 26–29. In 28 the egg has begun to divide at the side; the lobe contains the oil; in 26 the division is incomplete; in 27 the cleavage furrow begins near the antipole where the remains of the lobe containing most of the oil is present. No new lobe is formed opposite the cleavage furrow—i.e., at the true pole. In 29 the division was suppressed, the protoplasm is on opposite sides, the oil is in the lobe.

Eggs in the late two-cell stage were centrifuged for six minutes at 1,840 r.p.m. in reverse. The two cells were stratified independently of each other without altering to any great extent the shape of the cells (as shown in Plate II, 30), except in so far as the polar end of the larger cell, out of which the yolk was driven, became broader and the antipolar end more pointed—the reverse of the previous condition.

These results, with eggs about to divide, show the stability of the form of the egg during centrifuging in the jelly. This is perhaps to be expected, since the egg is held in a solid medium. On the other hand, when the gelatin is melted and the eggs are returned to sea water they retain the same form despite the new distribution of the interior materials. It appears that the form is determined by the surface layer that is affected very little or not at all by the centrifuging. The results show that the normal cleavage furrow fails to develop when the protoplasm and the mitotic figure are driven from the pole. Later a furrow may appear above the secondary position of the mitotic spindle. But in the last category there were no cases where the furrow cut through the center of the lobe of the egg, which remained at one side or was eliminated with the oil field. This indicates that the surface of the antipolar region of the egg is different from the surface of the rest of the egg, a conclusion in harmony with other results described in my previous paper.

THE SEPARATION OF THE EGG OF ILYANASSA INTO TWO PARTS BY CENTRIFUGING

T. H. MORGAN

(From the William G. Kerckhoff Laboratories of the Biological Sciences, California Institute of Technology, Pasadena)

Before giving the detailed experiments, in which the top of the egg (the polar region) is separated from the bottom (the antipolar region) by means of centrifuging in sugar solutions of various compositions, a few general points may be mentioned.

The relative size of the tops that come off depends in part on the condition or stage of the egg at the time of centrifuging, but largely on the medium in which the egg is centrifuged. In an isosmotic cane sugar solution, or even in stronger solutions, the tops are usually small. In a nearly saturated solution of raffinose the tops are generally quite large, and contain nearly all the protoplasm of the egg.

The spermatozoön enters the normal egg at any point on the surface. It is not easy to demonstrate the sperm-head in the newly laid egg, for as long as it lies at the surface of the egg it is quite small. At the time of extrusion of the polar bodies it is easier to find, since the sperm-head is larger, but it is still at the surface. The sperm-head enters the upper hemisphere more frequently than the lower. It does not sink deep into the protoplasm until after the second polar body is out. In newly laid eggs the germinal vesicle is sometimes present, but more often it is just breaking down when its large nucleolus is set free in the protoplasm and may, without some experience, be easily mistaken for the sperm-pronucleus at this time. The nucleolus disappears, however, before the sperm-nucleus approaches the egg-pronucleus.

In the centrifuged egg the sperm-nucleus is carried to the inner pole and lies in the protoplasm there, or on the border of the oil field. It is generally driven off with the protoplasm and oil when the top is large. I have seen very few cases where it is left with the bottom when a large part of the upper half of the egg has constricted off. Since the sperm-pronucleus may lie at first in any part of the egg, it is rather surprising that it appears always (?) to be carried to the centripetal end. In some other eggs, *Urechis*, for instance, the condensed sperm-head is heavier than the rest of the egg and is found most often near the pigment pole; but in *Ilyanassa* it is lighter and remains with the protoplasm. When a large top is thrown off it sometimes contains both the egg-chromatin and the sperm-pronucleus, since it gives off polar bodies and in many cases divides later. Such tops may, however, contain only the egg-chromatin. These give off polar bodies but do not divide. Here the sperm-nucleus appears to be absent, either because it is driven out of the top (with some of the oil), or because it is left behind in the bottom. Larger tops divide more often than smaller ones. Conversely, when only a small top is driven off, it may contain the sperm-nucleus, leaving the egg-pronucleus in the bottom. Under these conditions the bottom is not expected to divide, since it lacks the sperm-center. Such bottoms I have found in stained preparations. They produce lobes that are like the normal second lobe in every respect, and the lobe is better developed than the lobes of small bottoms that lack all chromatin. Many bottoms do not contain any of the egg-chromatin. Nevertheless, as will be described, they too form lobes. I have not succeeded in identifying any bottoms that had lost the egg-chromatin but retained the sperm-nucleus. Some such bottoms are perhaps to be expected, especially when the sperm happens to enter the antipolar hemisphere. I had hoped to identify monospermic fragments by the reduced number of chromosomes, but owing to the smallness of the chromosomes I have not succeeded in counting them. The possible occurrence of such bottoms does not affect the main issues raised here.

Centrifuging in Cane Sugar Solution

Eggs taken from a capsule that is just being laid were centrifuged at 2,680 r.p.m. for 9 minutes in a cane sugar solution consisting of 450 gm. sugar in one liter of tap water. The tops consisted of an oil cap, a clear zone, and a little fine yolk beneath (Plate I, 2). The tops were much smaller than the bottoms (Plate I, 1). In the control the second lobe began 17 minutes later, and in the tops the first polar body was coming off when removed, or a little later. It comes off usually at one side of the oil (Plate I, 4) or near its center. No lobes appear in the tops at any time. The controls divided 3 hours after the eggs were laid. Half of the tops divided at about this time into equal cells. They went into a four-cell stage one hour and 12 minutes later (Plate I, 6) and after one hour and 38 minutes into 8 cells with micromeres (Plate I, 7). The bottoms had gone to pieces (absorbed water) in this set.

In another set, just laid, the eggs were centrifuged in the same solution for 7 minutes at 2,680 r.p.m. The tops were larger than in the last set (compare Plate I, 14 and Plate I, 4). The bottoms were drawn out (Plate I, 8–9) with a neck containing clear protoplasm.

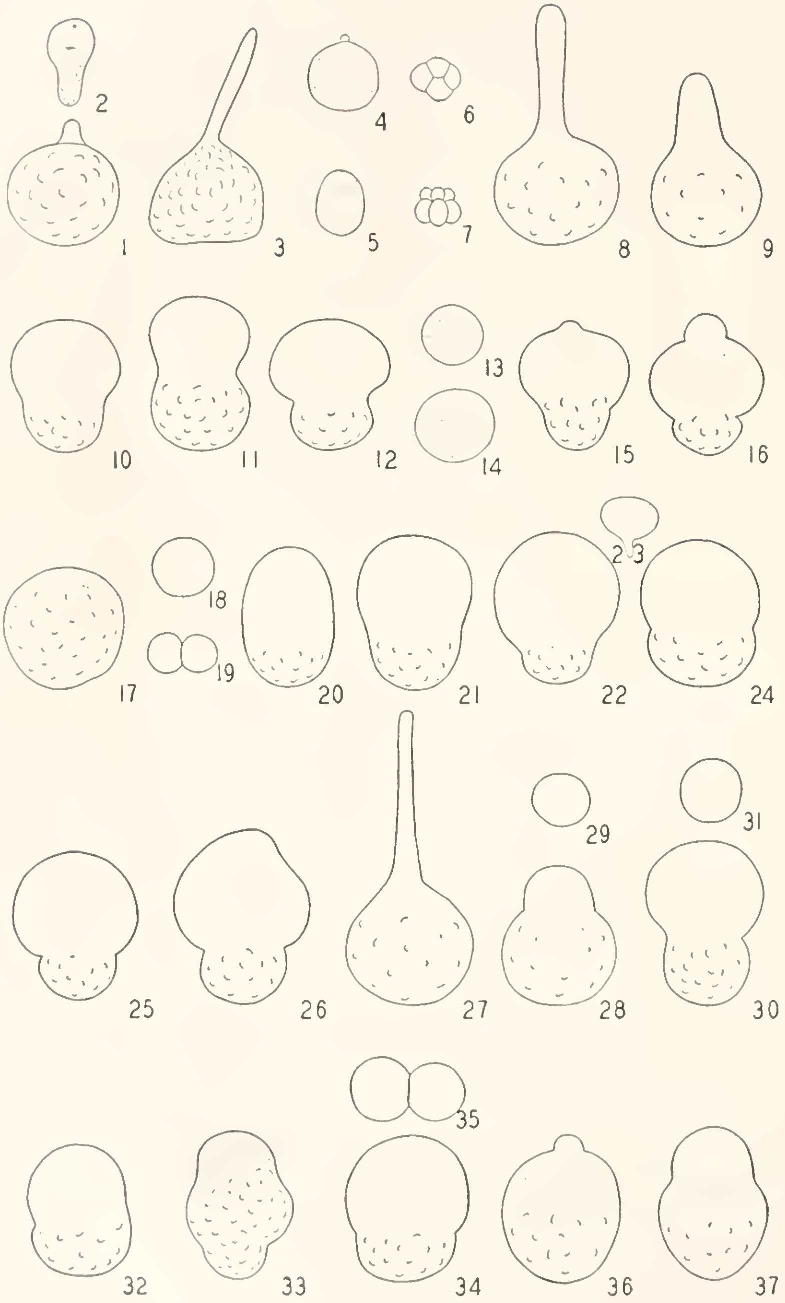

Plate I

Polar bodies came off from the tops; none from the bottoms. The tops divided after three hours and 45 minutes, when the controls were going into four cells. The bottoms, with no polar bodies or nuclei, elongated and formed a lobe-like bulge (Plate I, 10–12) in three hours and 42 minutes which partially returned in an hour. Later the bottoms assumed the shape drawn in Plate I, 15–16, with a lobe-like antipolar portion and a clear region at the opposite end with a nipple-like protrusion. No division took place.

In another set centrifuged for 8 minutes at more than 3,000 r.p.m. the tops were small and clear (Plate I, 18). The oil had been driven out of them. Two of these divided later (Plate I, 19) into two and then into four cells. The bottoms were relatively large and produced lobes (Plate I, 20–21).

Newly laid eggs were centrifuged one hour and 20 minutes before the second lobe appeared. The tops were few and small, Plate I, 23; the bottoms conspicuously large, Plate I, 22; and, as the sequel showed, half of them retained the nuclei, presumably both egg- and sperm-pronuclei. When removed it was evident that some protoplasm had remained with the bottom. All the bottoms developed a lobe, which later retreated both in those with and those without nuclei. In these bottoms, presumably the ones without nuclei, the lobe was less marked. Those without chromatin became spherical, and after two hours developed good lobes, Plate I, 24. Fifty minutes later they were spherical again, and an hour later lobes again appeared (Plate I, 25–26). Once more they became spherical, but were not further observed. It is obvious that when more of the protoplasm had been left in the bottoms they underwent a succession of lobe formations more conspicuously than when more of the protoplasm was removed. Stained preparations showed no elimination of chromosomes (i.e., polar bodies) in these bottoms.

In another set the tops were not found at the surface, but were in the outer end of the tube mixed with the bottom, and attached by thin threads to the yolk portion. The tops were no doubt about to separate. Some of the bottoms had long protoplasmic extensions (Plate I, 27) which retracted later. As the subsequent development showed, some of the bottoms segmented normally, although—and this is significant—they did not give off polar bodies. In the normal control, polar lobes were present one hour and 17 minutes after the other eggs had been centrifuged. The relative size of the tops and bottoms are shown in Plate I, 28–31. Of the tops, 7 divided, 11 did not; and of the bottoms, 8 did not divide and 17 did so. This gives approximately the ratio of the bottoms that retained the sperm-nucleus and those that

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did not. The bottoms that did not divide had lobes (Plate I, 32) present two hours later. At this time some of them showed a conspicuous accumulation of protoplasm at the polar end; others were spherical with a protoplasmic cap. The lobes of the bottoms slowly retracted; the bottoms remained in a spherical form for one hour, and after 25 minutes small lobes appeared on two of them but not on a third. Stained preparations (Plate I, 33) showed no chromatin present in these bottoms. The centrifuged eggs that had divided were then in the resting two-cell stage.

At the same time that the last set was centrifuged, eggs from the next capsules in series were rotated in the opposite arm of the centrifuge. The second lobe formed in these at about the same time as in the former. Many tops were present, and most of the bottoms were flattened and disintegrating (absorbing water). The few that remained are described below. They were smaller than the whole egg and developed lobes at the same time as the controls (one hour and 40 minutes after centrifuging). Forty-three minutes later they became spherical, and 40 minutes later lobes reappeared. Forty-five minutes later they were again spherical (one in division). Two of the tops divided at the same time, and 8 did not. Later, 5 tops were in four-cell and 4 had not divided, although polar bodies were on them, meaning that the egg-nucleus was present, but the sperm-nucleus had gone off with the oil. Forty minutes later the bottoms were spherical.

Several other sets were followed, but gave no results differing from the preceding ones. In some of the sets, 325 gm. sugar to one liter water was used; in others 374 gm. sugar. In general, when the bottoms divided, the tops did not, whether large or small, which means that the chromatin had remained in the bottoms and presumably the male pronucleus also.

A set of eggs was centrifuged for 5 minutes at 2,680 r.p.m. In the control the first polar body came off one hour and 27 minutes later. There were many tops. The bottoms had a protoplasmic extension when removed. About half of the bottoms gave off polar bodies. Lobes were present in the bottoms two hours and 36 minutes after centrifuging, and 30 minutes later some of them divided. The bottoms that did not divide had good lobes (Plate I, 34). One hour and 13 minutes later 8 tops had not divided and 7 had divided (Plate I, 35) meaning that the sperm-nucleus was absent in half of them. Of the bottoms, 31 divided (some with polar lobes), others not. Later, the latter became spherical; 3 tops divided into four-cell; 3 did not divide. An hour later the undivided bottoms had a protoplasmic zone (Plate I, 36–37) with a nipple-like protoplasmic extension, and an hour later, when killed, a broad zone of protoplasm, which, as shown in stained preparations, did not contain any chromatin.

CENTRIFUGING IN RAFFINOSE SOLUTIONS

In all later experiments a solution of raffinose was used in place of the cane sugar solution. At first 3.5 gm. of raffinose to 25 cc. tap water was used. Later 7 gm. was used, which gave better results. After dissolving the raffinose on a water bath at 45° C. it made a thick syrup. The eggs were added with a little sea water at the top of the small centrifuge tube filled with raffinose. In the 3.5 gm. and 5 gm. solutions the eggs sank to the bottom; in the 7 gm. solution they stayed at the top, but were driven to the bottom on centrifuging. The stronger solutions gave larger tops. Nearly all of these sets were centrifuged as soon as laid. After 10 minutes centrifuging at 2,500 r.p.m. the tops were floating near the surface or just below it. The bottoms were in the centrifugal end of the tube, and generally flattened against the glass. The tops were often as large as, or even larger than, the bottoms. There were individual differences in different sets due to the stage of the eggs when laid, and probably also to differences in the viscosity of the eggs of different individuals.

Eggs were centrifuged for 10 minutes at 2,500 r.p.m. and gave many rather small elongated tops that rounded up later (Plate II, 3). The second lobe developed after two hours in the control, and also in the large bottoms (Plate II, 2) but no bottoms showed polar bodies. An hour and a half later lobes appeared again (Plate II, 4). Half of these bottoms (16) cleaved, and half remained spherical without dividing. Twenty minutes later lobes were present on the bottoms that had not divided. Some of the tops had divided. Twenty-four bottoms in all cleaved. It is clear here that the pronuclei had not been driven off into the tops in most cases. It is also evident that, since no polar bodies came off either in those that divided later or in those that did not, the chromosome groups in one or the other kind of bottoms must have been tetraploid or pentaploid. The bottoms that divided presumably had both egg nucleus and sperm pronucleus.

In the following experiments a different solution of raffinose was used. To twelve drops of raffinose (7.5 grams of raffinose to 25 cc. of tap water) were added three drops of tap water. Experience showed that this mixture gave more and better tops.

When removed from the centrifuge many of the tops from the raffinose, like those from cane sugar, are elongated with pointed ends, but some of the tops are more rounded. Later, in sea water, they become spherical, or nearly so. Sometimes the middle of the elongating

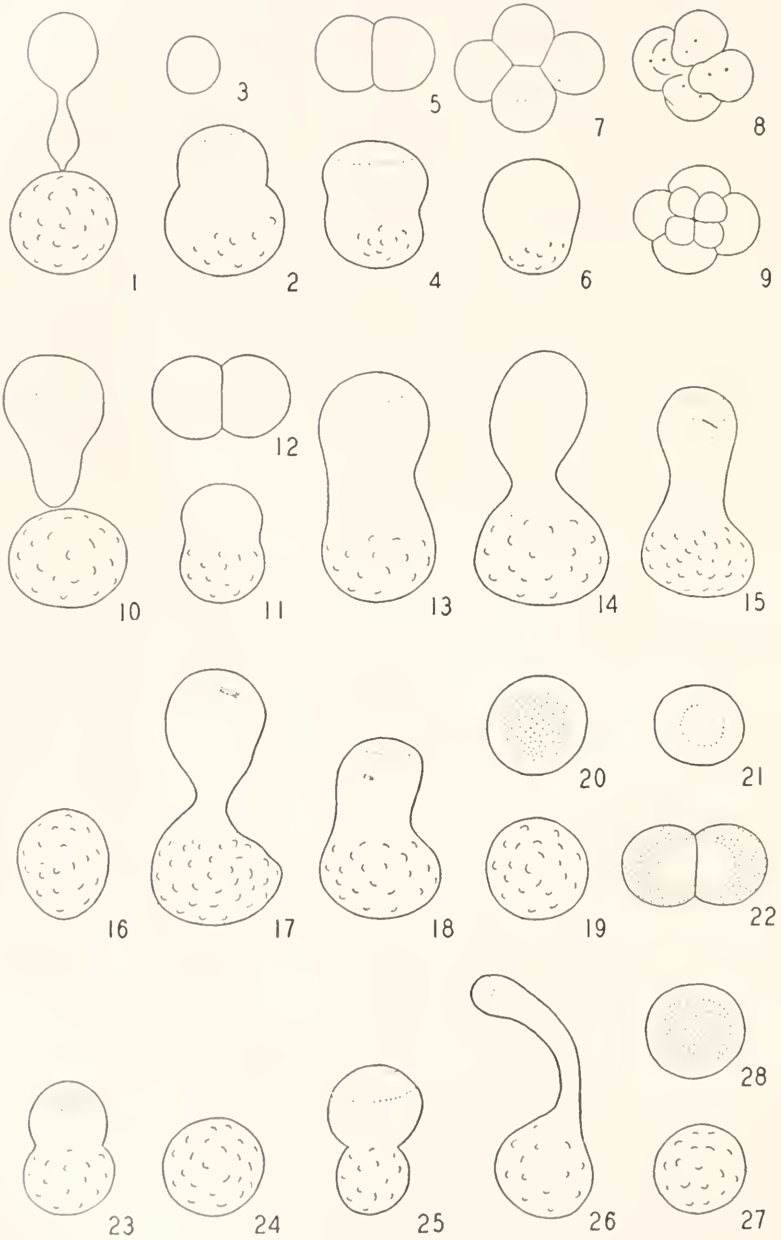
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Plate II

middle part of the egg also constricts off after the tops have separated, giving pieces of various sizes here spoken of as "middles." They contain some or all of the protoplasmic part of the egg. These also round up later. They contain no oil and often no nucleus, which goes with the tops. The tops contain all or part of the oil, and the more deeply stained protoplasm in which the chromosomes are frequently present. Rarely the sperm-nucleus can be identified in the tops. Generally it cannot be found. Since many of the tops divide later, there can be no doubt that the sperm-nucleus is in them. Some of the oil may be driven out of the top. A globule of oil is seen not infrequently outside the apex of the top. Of the many sets centrifuged in raffinose only a few typical examples need be described.

Eggs centrifuged for 10 minutes in raffinose at 2,500 r.p.m., thirty minutes before the second lobe, gave off tops that were nearly the same size as the bottoms (Plate II, 5–9). Some tops were not yet off when removed from the machine. Some of the tops divided later into two cells. Some of the larger bottoms that retained protoplasm and chromatin, but gave off no polar bodies, became lobed (Plate II, 6); others that were small without protoplasm remained spherical at this time. At the eight-cell stage the tops gave off micromeres, apparently in a dextrospiral (Plate II, 8–9). The small bottoms, when stained, were found to contain no protoplasm or chromatin at the apical end.

Another set of eggs was centrifuged for 10 minutes at 2,500 r.p.m. in raffinose. When removed, many of the tops were separating (Plate II, 10). Some of the tops were as big as the bottoms. Later 16 tops divided into two cells (Plate II, 12), and 8 did not divide. The bottoms were constricted in the middle at this time (Plate II, 11). Each had a slightly granular apical end. Stained preparations showed that there was no visible chromatin in the bottoms. The dividing tops (18) went into four equal cells and then into eight cells with micromeres. No lobes appeared in the tops. Ten did not divide. After 2 hours and 45 minutes the bottoms still showed the constrictions, i.e., they had not rounded up.

Another set, centrifuged for 5 minutes at 2,500 r.p.m., gave some good tops, but the separation had not been complete in some of the bottoms as seen alive (Plate II, 13–14). These show well the method of constricting the upper from the lower half of the egg. When preserved (Plate II, 15–18) a plate of chromosomes was found near the end of the clear protoplasm and beneath the oil.

Eggs centrifuged for 15 minutes at 2,500 r.p.m. gave nearly equal tops and bottoms. Nine tops went into two cells after 3 hours and fifteen minutes; 16 bottoms became constricted; others not. Thirty-five minutes later 12 tops were in two cells, 33 had not divided; 12 bottoms were constricted, 46 not. Forty minutes later 2 tops were in 4 cells; 16 bottoms were constricted; the rest were spherical. Twenty-one minutes later, 12 bottoms were constricted, the rest spherical, and 3 tops were in the four-cell stage. Thirty minutes later, 3 bottoms were slightly constricted, the rest spherical; the tops were in four cells. Nineteen minutes later 2 tops were in eight cells; one was still in two cells; the bottoms were as before.

Eggs centrifuged for 10 minutes gave large tops that were about the same size as the bottoms (Plate II, 19–21). A few bottoms had not pinched off from the tops (Plate II, 23, 25, 26). One and a half hours later, 20 tops had polar bodies; the larger bottoms (without polar bodies) had lobes; 16 middles had no oil and no polar bodies; 22 small bottoms were spherical. Two and one-half hours later 13 tops were in two cells (Plate II, 22), and the bottoms were constricted; the latter showed some protoplasmic material at the apical end. An hour later the bottoms were still constricted and, after another 30 minutes, 20 tops were in four cells; 8 big ones had not divided; one middle had divided into three cells; the smaller middles had not divided. At this time 5 bottoms were spherical and 2 were constricted.

Centrifuged in Sea Water

Newly laid eggs were taken from the capsule and centrifuged in sea water at 2,500 r.p.m. for 5 minutes, and again for 4 minutes. They were flattened on the bottom and stratified, but the tops did not come off. The yolk burst when an attempt was made to dislodge the eggs. Another lot was centrifuged at more than 3,000 r.p.m. for 10 minutes; the eggs were stuck to the glass and could not be removed even after an hour. No tops had come off.

Centrifuged Inside the Capsule

Inside the capsule the eggs are imbedded in a sticky jelly. When centrifuged in the capsule the eggs collect in a plate of cells, and if crowded become columnar in shape. The oil comes off as small globules that do not contain chromatin. They float to the end of the capsule opposite the mass of eggs and, when the capsule is opened, to the top of the water. None of these oil-tops divide.

Newly laid eggs were centrifuged inside the capsule for 12 minutes at about 3,000 r.p.m. The capsule was opened under picrosulphuric acid. Most of the bottoms were stuck to the wall and came off in a lump. The stained bottoms had a clear outer end (stained blue). The chromatin was in this region. This "middle" part, if pinched off or broken off, contained a metaphase spindle.

Other similar eggs were centrifuged for 10 minutes at more than 3,000 r.p.m. All were in a lump at the bottom of the capsule, arranged in a palisade, with inner pointed clear ends. Sixty oil-tops were counted. The capsule was opened after 1½ hours. Many large middles were found which an hour later divided into two equal cells. Some of the bottoms did not divide; others divided.

Eggs were centrifuged in the capsule for 10 minutes at 2,980 r.p.m., and then, since only some of the oil-tops were off, for 5 minutes more. All of the oil-tops were off, i.e., that region from which the normal polar bodies come, and the bottoms were flattened against the capsule, and against each other. After about an hour the capsule was opened. After another hour nearly all the middles were pinching off (Plate III, 1–5). Each contained a little oil, the rest was clear protoplasm. Seven minutes later these were killed and stained. Except for one middle, no polar bodies were present, but a large nucleus, or two or three smaller ones, was present in the protoplasm (Plate III, 1–4), or in the neck of the protoplasm. These late-developing middles might be regarded as polar bodies, but if so it is only because some of the chromatin at the time when the polar bodies are due to develop happens to lie within the protrusion of protoplasm that may or may not pinch off. In fact, all the chromatin may come to lie in the protrusion as a large vesicle. It is probable that when these bottoms were killed the first division was about to come on.

Eggs were centrifuged for 10 minutes at 2,500 r.p.m. The oil tops were off most of the eggs. One hour and twenty minutes later the eggs showed the second lobe; some had one polar body out (Plate III, 10); others did not show any polar body (Plate III, 9), and others had a clear middle still attached (Plate III, 6–8). An hour and 23 minutes later the eggs were about to divide (Plate III, 6–7). The yolk lobe was constricted. Some eggs had large middles attached. Eighteen minutes later the eggs were about to divide, and were killed and stained. Some of the eggs divided regularly into two cells with a lobe: one had two polar bodies; one egg had a dividing nucleus, half in the middle, half in the bottom. If this is a cleavage figure, the middle is a blastomere rather than a polar body. Another divided egg had a middle (with a faint mass of chromatin) which might possibly be called a polar body.

Eggs were centrifuged for ten minutes at 2,980 r.p.m. The oil-tops came off. The bottoms were compressed into hexagons against the wall of the capsule. When they had rounded somewhat the capsule was opened (one hour and 13 minutes later). No polar bodies were seen

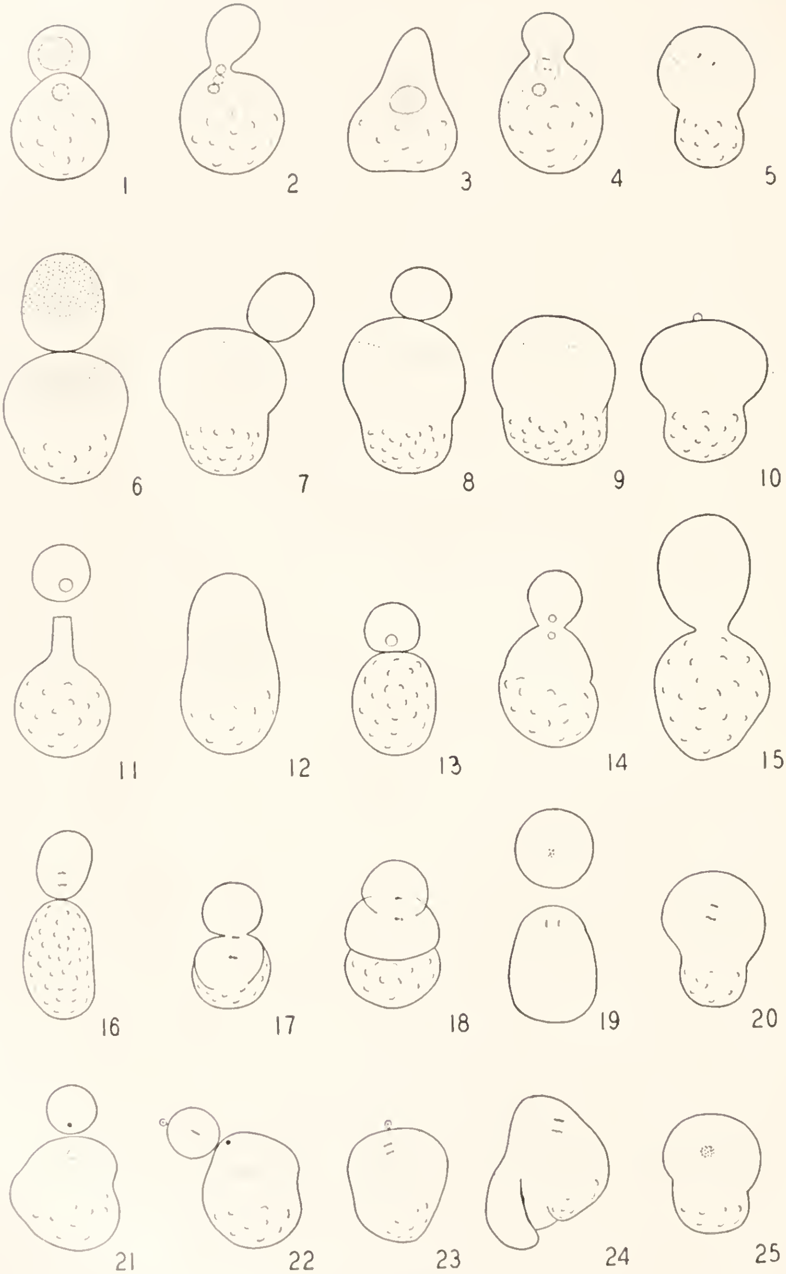
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Plate III

except in two eggs. Several middles constricted off (Plate III, 11–15). Each contained a nucleus (Plate III, 11, 13, 15). In some of the bottoms a clear middle was still partly constricted off and may have contained a nucleus in only the clear part. Possibly these middles, too, might be interpreted as polar bodies.

Centrifuged for 10 minutes at more than 3,000 r.p.m. many eggs after two hours constricted off the middles (Plate III, 16–20). Some middles were entirely free, others were attached. The second lobe was present in eggs killed two hours after centrifuging (Plate III, 20). No polar bodies were given off, but the second anaphase spindle was present in most eggs. Metaphase spindles were present either in the middles (Plate III, 16), or in the constriction between the middle and the bottom (Plate III, 17–18), or in the bottom (Plate III, 19). Whether these middles should be called polar bodies seems doubtful.

In one set centrifuged for 20 minutes at 2,680 r.p.m. the eggs were compressed into three clumps against the capsule. An hour later they had separated somewhat, and two hours later were removed from the capsule, killed, and stained. In all of them the second lobe was present. The condition of the eggs is shown in Plate III, 21–25. Most eggs had no free polar bodies. Several had attached middles. Two of these had chromatin in them. The second spindle, in anaphase, was present in some of the eggs. One egg had a first polar body attached to the middle (Plate III, 22). In another it was attached to the egg (Plate III, 23) and below it an anaphase spindle.

Conclusions

The preceding experiments, concerning the development of the second and third lobes in eggs from which the tops or middles have been centrifuged off, may be compared with the rhythmic behavior of the isolated third lobe described in a preceding paper (1933). There are several categories of cases. More or less of the protoplasm may be present in the tops or in the middles. Accordingly, the sperm-nucleus and the egg-nucleus may be both removed from the bottoms. Again, only the sperm-nucleus may be removed and the chromatin of the egg-nucleus may remain in the bottom. This chromatin may consist of only the reduced egg-chromatin (if the polar bodies have been given off), or the egg-chromatin may have divided in the egg without being reduced by the extrusion of the polar bodies (giving a triploid, tetraploid or pentaploid group). There is the further possible case, viz., the egg-chromatin being thrown out in the top while the sperm-nucleus remains in the bottom.

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The evidence concerning the lobe is explicit. The second lobe develops, whether or not polar bodies are extruded, in the presence of the egg-chromatin alone. Furthermore, the evidence shows that the second lobe develops even when all the chromatin has been thrown out of the bottom. It is true the lobe appears less well developed when all the chromatin is absent, but even then it appears and its less conspicuous formation may depend on the smaller size of the bottom.

The results also show that there is a rhythmic series of changes in the bottoms. The second lobe is withdrawn and the bottom is spherical again; then the lobe appears at approximately the same time (or is somewhat delayed) when the third lobe develops in the control. The alternate appearance and disappearance of the lobe has not been followed so far or in as much detail as in the cases described briefly in my former paper, in which the isolated third lobe only was studied. The latter situation appears in some respects a more interesting case, since it is the next to the last stage when the yolk lobe appears.

Discussion

Since most of the problems connected with the experiments described here have been mentioned in the text, it does not seem necessary to go over the ground again. A few points may, however, be mentioned that involve comparisons between the behavior of the separated lobe-end of the egg (the "bottoms") obtained by centrifuging and those obtained by isolating the third lobe after it is constricted from the egg. Since the former are obtained before the polar-bodies are set free, and the latter after the first cleavage is completed, it is possible that the antipolar region may have undergone certain progressive changes during the interval. In fact, the behavior of the two regions is somewhat different. For instance: when the third lobe is shaken off, it undergoes a series of rhythmic changes described in the preceding paper. Wilson, who first observed the same phenomenon in the isolated lobe of *Dentalium*, suggested that the isolated lobe formed a lobe on itself. I have pointed out that since the isolated lobe may continue to repeat this several times (more times than does the normal egg), it is possible that the constrictions do not represent so much the repetition of lobe formation as they do the change that this part of the egg undergoes after it becomes incorporated in the *D* blastomere.

When the rhythmic changes in form of the isolated third lobe are compared with the changes in shape of the non-nucleated bottoms obtained by centrifuging, it is clear that the bottoms undergo rhythmic changes that resemble to some extent those of the isolated third lobe.

But there are differences, and in fact such would be expected, since the bottom might (or might not?) be expected to form a lobe three times in succession, each of different character from the others. If the first lobe formed in such bottoms it might pass unnoticed. It was not observed. If the second lobe formed, it should be quite conspicuous, but like the normal second it would not be expected to pinch off. If the third lobe formed it might be expected to completely pinch off. Nothing of the sort was observed. On the contrary, the succession of changes in the bottoms is not so conspicuous, and perhaps not so prolonged as those of the isolated third lobe.

A comparison should also take into account the possibility of progressive changes in the whole egg during and after polar body formation. In the absence of the polar hemisphere in the bottoms the changes that normally occur in that hemisphere may be restricted or lessened, while these changes may have happened by the time the third lobe is formed; hence comparisons would scarcely be worth making.

The fact that the lobe forms when its interior material has been driven out shows that the formation of the lobe is not dependent on its interior materials. Since it develops approximately at the same time as does the lobe in the whole egg, namely, when the mitotic figure has reached a certain phase, and when the mitotic figure may be in a part of the egg (at the side, or even in the antipolar hemisphere itself) different from its position in the normal egg, it follows that lobe formation, as such, is not a by-product of the mitotic figure, but that the development of the two represent synchronous and more or less independent changes in the egg. Since the interior of the lobe in the inverted eggs may be composed in part of oil and in part of protoplasm it seems to follow that it is in the surface layers, not disturbed by centrifuging, that the cause of the phenomenon is to be sought. This statement is not in conflict with the fact that the complete pinching off of the third lobe is due in part to the cleaving of the polar hemisphere ino two equal parts, leaving the lobe outside (see below). The more complete isolation of the lobe region at the time of the first cleavage is due to the rounding up of the blastomeres, which is a phenomenon superimposed on the lobe-formation.

The cleavage of the tops, both small and large, into two equal parts, then into four equal parts, and then into the unequal micromere division, invites comparison with the results obtained with fragments of the eggs of *Chaetopterus* described by Wilson and by Whitaker and Morgan. In *Chaetopterus* the first division of the fragment is unequal, as it is in the normal egg, although the polar lobe of this egg is very small in comparison with that of *Ilyanassa*. Evidently the inequality in the *Chae-*

topterus egg is not due to the presence of an antipolar lobe region through which the first division can not pass. In *Ilyanassa*, on the contrary, the first cleavage of the normal egg is into equal parts in the polar hemisphere, and gives the impression of passing to one side of the antipolar region as though the cleavage plane could not pass through it; but the equal division of the top shows that the inequality in *Ilyanassa* is not a primary inequality as in *Chaetopterus*, but on the contrary the division is equal or nearly so. The subsequent fusion of the lobe with one blastomere is responsible for the apparent inequality of this division.

In both animals fragments of the polar hemisphere do not form lobes, while the antipolar hemispheres do produce lobes. In this connection a further question arises. Is the first division across the antero-posterior axis of these eggs? Does such a condition pre-exist in fact, or develop only during or after the division? I do not know of any decisive answer to this question. Even in the eggs of the frog, of *Amphioxus*, or of *Styela* it has not been convincingly proven, in my opinion, that an antero-posterior axis exists prior to fertilization. After that event it certainly appears to be present. So important is the decision on this point that I shall remain skeptical until more convincing evidence is produced. If it is true that such an axis exists, its presence would seem to simplify some of our difficulties of interpretation, but the wish to have the question settled should not affect our attitude in regard to the evidence.

So far as the location of the lobe before fertilization is concerned, it might appear that, if there is a preformed antero-posterior structure to the egg, the incorporation of the lobe into one only of the first two blastomeres could be explained as due to some peculiarity of the surface layer on one side that prevents one blastomere from completely cutting off—hence the lobe remains as part of one blastomere (the dorsal one) only. But attractive as such an assumption might appear, it is also possible that just before cleavage an antero-posterior change takes place on one side of the egg which is responsible for the failure of the lobe to pinch off on that side. The whole question of the relation of the sperm entrance and the plane of fusion of the pronuclei is concerned in this question; and it is better, I think, not to prejudge it, even though it may seem to offer a simple way out of a difficult problem to assume that a preformed dorsal side pre-exists. If, as has been shown, there is a definite relation between the entrance point or penetration path of the sperm and the establishment of an antero-posterior axis, then to assume a preformed axis to solve formally one problem may land one in a contradiction with respect to the other problem. If, however, the antero-posterior difference comes in after fertilization, both problems may find a common solution.

Towards the end of the experiment several cases were observed in which the first or second polar spindle lay at the base of a protoplasmic protrusion of the bottom (Plate III, 17, 18, 14). Into this protrusion one or more polar nuclei may pass. The protrusion may later pinch off. It might then be called, with reservations, a giant polar body and compared with a somewhat similar process Conklin has described for *Crepidula*. Without a fuller series of observations on *Ilyanassa* I do not feel like deciding whether the isolated "middles" are comparable to polar body formation, or whether there is something here *sui generis* owing to the very unusual conditions that are present at the time.

THE RHYTHMIC CHANGES IN FORM OF THE ISOLATED ANTIPOLAR LOBE OF ILYANASSA

T. H. MORGAN

(*From the William G. Kerckhoff Laboratories of the Biological Sciences, California Institute of Technology, Pasadena, California*)

In a previous paper (1933) I have described the rhythmic changes in shape of the third yolk lobe when isolated. In the summer of 1934 I made at Woods Hole the additional observations described below.

The simplest way to separate the third lobe from the two blastomeres is to squirt the egg gently in and out of a pipette at the time when the lobe has pinched off from the two cells. It often comes off when the eggs are removed from the capsule if they are in the two-cell stage. It is noticeable that it is less often injured than are the two blastomeres which, when shaken off in the two-cell stage, often go to pieces at once. The isolated lobe soon becomes spherical. Sometimes, however, it too absorbs water, swells up and dies.

The following record gives the history of the isolated third lobes. It is not easy to give an account of the change that takes place in the isolated lobes without going into great detail. The lobes were followed continuously for several hours and sketches made. I have selected four records out of twelve, which tell sufficiently the story with the aid of the drawings.

Case 1

The two-cell stage was shaken at 12:05 P.M.; at 2:25 the isolated lobes were oval (Figs. 1, 2); at 2:50 pointed or with a nipple (Figs. 3, 4). They were round at 3:13 (Fig. 5), blunt at 3:50, pear-shaped at 4:08 (Figs. 6, 7, 8); more constricted at 4:17 (Figs. 9, 10, 11), and at 4:37; round at 4:53 (Fig. 12); round or elongated at 5:37 (Fig. 15); constricted at 5:45 (Figs. 14, 15, 16).

Summary: The lobe became oval or pointed when the controls were in 4-cell stages, and constricted again when the controls were in 16-cell stages. They then became round, and again constricted. There is no obvious correlation between the times of cleavage of the control and the alternate phases of constriction of the lobes.

Case 2

The 2-cell stage was broken apart by squirting in a pipette. The lobes were slightly pointed at 12:14 (Fig. 17); the lobes rounded up at

12:20, were oval at 12:38 (Fig. 18), and at 12:52 to 2:40 they were round (Fig. 19). At 3:05 to 3:21 (Figs. 20, 21, 22, 23, 24) they became pointed, then constricted (the whole eggs were going into 8-cell stages). At 3:40 they were round (Fig. 25) until 4:15 to 4:43 when they became constricted (Figs. 26–32). At 5:15 the lobes were spherical. Summary: Here the lobes became constricted twice, much more slowly than the cleavages of the normal control.

Case 9

Lobes were shaken off at 11:55. They were round at 12:03 and remained so until 1:53. At 2:56 (Fig. 33) they became constricted (the normals were in the 4-cell stage). At 3:48 the lobes were oval (Fig. 34), one was round; at 4:14 they were pear-shaped or slightly constricted (Figs. 35, 36); at 4:45 they were spherical (Fig. 37); at 5:24 elongated (Fig. 38) with clear ends. At 6:22 they were oval or round (Figs. 39, 40); at 7:13 to 7:55 (Figs. 41, 42) they had clear ends with a constriction. At 8:52 to 9:18 they were round; at 8:52 they were still round, and at 9:50 (Figs. 43, 44) they were oval or with a slight constriction. Summary: In this case there was a succession of changes in shape and an accumulation of clear protoplasm at one end. The changes were not obviously synchronous with the changes in the cleaving eggs.

Case 11

Shaken off at 2:35, the lobes were round at 2:55. At 3:35 some were oval with a clear nipple (Fig. 45); others pear-shaped. At 3:46 they were round (Fig. 46); at 4:11 to 4:42 most of the lobes were oval with clear ends (Fig. 47); at 5:22 they were round again (Fig. 48); at 6:19 they were pear-shaped (Fig. 49); at 7:11 they were round (Fig. 50). At 7:50 they were oval and elongated (Figs. 51, 52); at 8:22 they were round; at 9:15 most were round (Fig. 54), some with a clear end (Fig. 55) which was still present at 9:53 (Fig. 56).

The question now arises as to whether these changes in the form of the lobes is to be interpreted to mean that the isolated lobe continues alternately to form a succession of lobes, or whether the changes in shape represent some other kind of rhythmic activity. I have discussed this question in my previous paper and raised there certain theoretical objections to the literal interpretation of the changes in shape as repetitions of lobe-formation, chiefly because these isolated third lobes may go on alternately constricting and rounding up more times than this region does when it is a part of the normal egg.

The experiments, reported in other papers in which the egg is constricted into two parts by centrifuging in raffinose, reopen the question of the interpretation of the rhythmic changes in the "lower" half (antipolar hemisphere) of the eggs there spoken of as the bottoms. These experiments show clearly that the "upper" polar half of the egg,

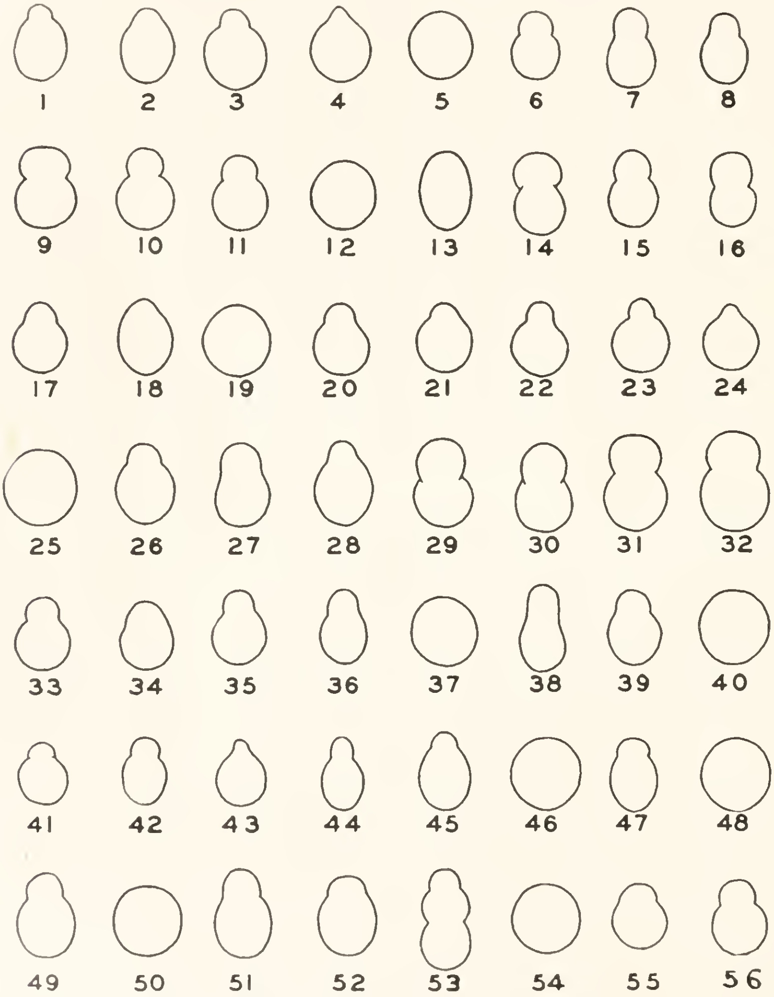
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Plate I

that does not contain the lobe-forming portion of the whole egg, does not repeat the type of cleavage of the whole egg since no lobe appears on it, either before or after the first cleavage. Again, it is clear that the antipolar half or bottom, which contains all of the lobe region, develops a constriction at least twice, whether it contains the egg-chromatin, or whether none of it is present. Since these two changes are vaguely synchronous with the lobe-formation of the whole egg, they appear to correspond to that phase. If this is correct, it reopens the discussion of the changes in shape of the isolated third lobe.

That the changes in the third lobe are not strictly synchronous with the second cleavage is apparent—they are much delayed. This in itself might not rule out the interpretation that they represent lobes, but it is to be remembered that the lobe appears only once more after the first cleavage ends, while the isolated third lobes come and go at least three times. It should also be remembered that, strictly speaking, the change in shape does not give a reduced picture of the whole egg and its lobe, as a comparison of the figures illustrates. Furthermore, after the second cleavage, the antipolar hemisphere normally becomes incorporated in one of the first four cells, and at the next cleavage this cell pinches off a micromere from the apical end. The changes in shape of the isolated third lobe resemble somewhat more this process of constricting off micromeres than they resemble the lobe-formation of the early stages. I am inclined, therefore, towards the same interpretation of the later rhythmic changes of the isolated third lobe, rather than the interpretation that lobe-formation repeats itself several times. There is also a general consideration that should be given some weight. The cleavage stages are progressive steps. Even isolated blastomeres carry out the partial pattern rather than returning to the previous stage. Hence I think we might expect the rhythmic changes of the third lobe to do this also. If so, the lobe-constrictions and concentration of clear material at the polar end correspond in a way to the later development that this part normally undergoes if it remains in the egg.

Returning again to the centrifuging experiments, the behavior of the bottoms may be interpreted to mean that they represent the two normal stages of lobe formation. The later phases that appear would then be interpreted in the same way as those of the isolated third lobe. There is, then, no inconsistency in interpreting the bottoms obtained by centrifuging eggs in an early phase and the interpretation of the rhythmic changes of an isolated third lobe as representing later stages of development, since the latter begin at a later period. The interesting fact to be emphasized is that this part of the egg deprived of a nucleus continues to undergo rhythmic changes resembling more or less the changes that it normally undergoes as part of the whole egg. In other words: there resides in the protoplasm of the mature egg the property of taking part in the progressive series of changes of the egg independently, to some extent, of influences depending on the activity of the genes at that time, or the presence of mitotic developments.

MODIFICATION OF MAMMALIAN SEXUAL CYCLES

II. Effects upon Young Male Ferrets (Putorius vulgaris) of Constant Eight and One-half Hour Days and of Six Hours of Illumination after Dark, Between November and June[1]

THOMAS HUME BISSONNETTE
Trinity College, Hartford, Connecticut

Introduction

Some experiments with young male ferrets in their first autumn, with six hours of electric light added after dark, led to some increase of spermatogenic activity, to complete activation of testis interstitial cells and of secondary sex-organs and to increased libido, leading to sterile matings on December 10, December 12, and December 21 with females brought into œstrus by similar increases of daily light exposures beginning on October 12 (Bissonnette, 1932). They did not give a clear picture of the differences between the sex-organs of such males on increased lighting and those of similar animals on a constant reduced day.

The rather preliminary study to be reported here was an attempt to test the effects of a constant 8½ hour day in autumn and winter as contrasted with those of " long days " consisting of normal days for the seasons to which 6 hours of electric lighting from an incandescent bulb was added after dark each night, in order to learn whether either of these alterations in day-length modifies the sexual cycles of such young males and, if so, to what degrees.

Material and Methods

On November 10, 1932, six males in their first year were placed in separate cages in two vertical rows, 1, 3, 5, in one series and 2, 4, 6, in the other, facing the light from three large windows of a basement room at a distance of about 7–8 feet.

The left-hand series (1, 3, 5) also faced a 300-watt clear bulb at such distances that the nestbox in cage 1 received light varying from

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[1] Aided by a grant from the Committee for Research in Problems of Sex of the National Research Council, 1932-33. Library and laboratory facilities were also furnished by the Marine Biological Laboratory at Woods Hole, Mass.

16.86 foot candles, nearest the bulb, to 9.25 at the back of the box; cage 3 received 16.31 to 8.81 foot candles; and cage 5, from 12.88 to 7.17 foot candles. Light from the windows was not measured, but fluctuated from day to day and with the season. The animals in these cages received decreasing daily daylight periods till December 21 and increasing ones thereafter till June 9. The decrease was about nine-tenths of an hour from 10 hours on November 10 to 9.1 hours on December 21. The increase was about 1.9 hours to February 24 and about 6.1 hours to June 9, when the last two experimentally lighted males were sacrificed. They were also subject to change in daylight intensity partly due to change in the height of the sun above the southern horizon during the day, reducing it gradually till December 21 and increasing it thereafter, partly to changes in daily sunshine.

The right-hand series (2, 4, 6) bore the same relations to the windows and to daylight intensity, but they were covered and shielded from light by a heavy carpet from 4.30 P.M. to 8.00 A.M. daily, during part of which time the electric light was shining into the other series of cages. This kept their day-length approximately constant at 8½ hours per day.

All the animals were behind ordinary window glass throughout the experiment and the light bulbs emitted only negligible ultra-violet radiation.

Food for all consisted of pasteurized milk till about May 1, and natural milk with cod-liver oil concentrates added thereafter, to which were added dog-biscuit and very occasional meat scraps, both raw and cooked, throughout the experiment. The physical conditions of the animals throughout were about normal, though one or two developed light attacks of "fōot-rot" before the whole milk and C.L.O. concentrates were introduced into the diet. However, this condition had been found in the previous experiments to interfere in no way with normal or induced sexual activity in these animals.

The position of the light bulb (similar to that used before) was such that it shone most horizontally upon the animal in the upper cage, more and more obliquely upon the middle and lower cages respectively, with distances greater in the lower ones. As noted before, this permits the ferret curled up to sleep to shield his head and eyes from light better in the upper cage than in the lower ones. Such shielding of the eyes was correlated with slower rate of activation sometimes, in spite of the shorter distance from the light and consequent greater luminous intensity at the upper levels (Bissonnette, 1932).

The amount of hair over the scrotum, and the conditions and positions of the testes were tested from time to time by palpation and inspection. Sexual libido, copulating power, and sperm ejaculations were also tested at various times as œstrous females were available from a parallel experiment on the effects of hoodwinking these animals and using hoods with eyeholes to learn the avenue of reception of the light stimulus to be described elsewhere. These trials began on January 13 and continued till June. On the former date none of the "short day" males showed any interest in copulating or even mauling an œstrous female. Of the "long day" animals, No. 5 made feeble attempts to copulate, No. 1 made very determined attempts but was unsuccessful in becoming "fast" to her; No. 3 succeeded and remained "fast" to her for 1¾ hours that day and again for 1½ hours the next day. These matings resulted in pseudopregnancy only, as there were no sperms in his ejaculations, as determined by microscopical examination of the fluid drawn off from her vestibule immediately after copulation ceased.

At killing, genital tracts, thyroids, and adrenals were fixed in Bouin's fluid and the hypophyses in Zenker's fluid, with formic acid replacing acetic in both solutions. After the usual paraffin technique, testes and epididymides were sectioned at 10 microns, stained in Heidenhain's iron-hematoxylin and eosin, and mounted in balsam. The other endocrine glands were saved with the rest of the genitalia for later study.

KEY FOR TABLE I

Copulations

⊕ − = tried feebly and unsuccessfully.
+ − = tried forcefully and unsuccessfully.
+ + = tried successfully.
− − = did not even try to copulate.

Sperms in matings

− − = sperms absent.
+N = sperms present, inactive, and few.
+A = sperms present, active, and few.
+ +A = sperms numerous and active.

Position of testis

A = anterior in groin, near winter position.
P = posterior in scrotum, near anus.

Pregnancy or pseudo-pregnancy

− − = no effect noted.
pP = pseudo-pregnant.
P = pregnant.

No. of animal

1, 3, 5 were exposed to added electric light.
2, 4, 6 were restricted to an 8½-hour day.

TABLE I

Data on sexual activity and behavior of "short day" and "long day" males

No. of male	Date	Copulation reaction	Copulation time *hours*	Sperms in matings	Testis size	Testis position	Pregnancy or not	Killed. Remarks.
1	Nov. 10	—	—	—	—	—	—	Light began
	Jan. 13	⊕ −	—	—	large	P	—	—
	" 14	+ −	—	—	"	P	—	—
	Feb. 21	+ −	—	—	"	P	—	—
	" 22	+ +	1	—	"	P	—	—
	" 23	− −	—	—	"	P	—	Killed Feb. 24
3	Jan. 13	+ +	1¾	− −	large	P	pP	—
	" 14	+ +	1½	− −	"	P	—	—
	Feb. 20	+ +	2	+A	"	P	P	Female gave young, Apr. 3
	" 21	+ +	—	+ +A	"	P	P	Same female
	Mar. 7	+ +	—	+ +A	"	P	P	Female died Mar. 31; 11 embryos
	" 11	+ +	—	+ +A	"	P	P	—
	" 18	+ +	—	+ +A	"	P	P	—
	Apr. 20	+ +	1¼	—	small	P	pP	Eggs shed before 36 hrs. after finish
	" 28	− −	—	—	"	—	—	—
	June 8	+ +	1	− −	"	A	—	Killed June 9: in regression

TABLE I—*Continued*

No. of male	Date	Copulation reaction	Copulation time	Sperms in matings	Testis size	Testis position	Pregnancy or not	Killed. Remarks.
			hours					
5	Jan. 13	⊕ –	—	—	small	A	—	———
	" 14	– –	—	—	"	A	—	———
	Feb. 20	—	—	—	"	P	—	———
	" 23	++	$2\frac{1}{4}$	—	medium	P	—	———
	Mar. 6	++	—	—	larger	P	—	———
	" 7	⊕ –	—	—	"	P	—	———
	" 8	++	—	—	large	P	—	———
	" 11	++	—	—	"	P	—	———
	" 13	++	—	—	"	P	—	———
	" 18	++	—	—	"	P	—	———
	Apr. 28	—	—	—	small	1A, 1P	—	———
	June 8	++	$1\frac{9}{60}$	—	"	A	pP	Ovulation 35 hrs. later
	" 8	—	—	—	—	—	—	Killed
2	Jan. 13, 14	– –	—	—	small	A	—	———
	Feb. 20	– –	—	—	"	A	—	———
	Mar. 8	++	—	—	larger	P	—	Killed Mar. 15
4	Jan. 13, 14	– –	—	—	small	A	—	———
	Feb. 20	– –	—	—	"	A	—	Killed Feb. 24
6	Jan. 13, 14	– –	—	—	small	A	—	———
	Feb. 20	⊕ –	—	—	large	P	—	Scrotum getting bare
	Mar. 7	++	—	+N	"	P	—	———
	" 12	++	—	++A	"	P	—	———
	" 19	++	—	++A	"	P	P	———
	Apr. 8	—	—	—	—	—	—	Killed

Results and Observations

Changes in Macroscopic Conditions and in Behavior

About two weeks after lighting began the testes of the "long day" animals (1, 3, 5) started to move backward into a position in the scrotum immediately below the anus and to enlarge and become turgid. The hair then thinned out over the testes till, at time of greatest testis size, the scrotum became almost bare. In this No. 5 lagged considerably behind the other two. In one of the "short day" males this was not delayed as much as in No. 5; but in the other two (2, 4) it was greatly retarded.

The results of these tests are summarized in Table I in detail.

Of the "short day" animals, No. 4 was killed for study on February 24 without showing any sexual libido at all; No. 2 showed sexual libido first on March 8, when he succeeded in copulating but probably without sperm ejaculation (a few non-motile sperms were found in the vestibule of the female, probably from a previous mating with another male giving abundant sperms) and was sacrificed for study on March 15 without conclusive evidence of sperm ejaculation; No. 6 first showed libido enough to try to copulate on February 20, unsuccessfully. He first succeeded on March 7 and left a few non-motile sperms in the vestibule of the female. By March 12 and 19 he was able to give abundant motile sperms in matings. He was killed for study on April 18 when his testes were still at the climax of activity.

Of the "long day" animals, No. 3 was most quickly stimulated and copulated without sperm ejaculation as early as January 13 and 14, and with abundant active sperms by February 20 and 24, inducing pregnancy on the twentieth. However, by April 20, his testes were smaller and soft, and a mating on that date was without sperm ejaculation, though it induced ovulation of the female within 36 hours after the finish of copulation and the granulosa cells had already left the eggs which were well down the Fallopian tubes. He was still farther in regression by April 28 and June 8; though on the latter date he copulated, without sperm ejaculation.

No. 1 was somewhat slower to respond and tried hard but unsuccessfully to copulate on January 13; was successful on February 22, but not on the twenty-fourth, when he was sacrificed (no test was made for sperms as the female had already mated with a male giving abundant active sperms).

No. 5 was slowest to respond. He made only feeble attempts to copulate on January 13 and 14; was successful on February 23, March 5, 8, 11, 13, and 15, but always without sperm ejaculation, so far as could be determined by microscopical examination of fluid from the vestibule of the females after copulation. By April 28, his testes were again small and one forward in the groin. But on June 8 he copulated for an hour and nine minutes with induction of ovulation within 35 hours of the finish of copulation, though his testes were both forward in the groin and small.

These facts indicate that libido is restored before complete potency for copulation, and both before complete spermatogenesis, but that spermatogenesis regresses before libido and potency to copulate. The copulations on June 8 by males No. 3 and No. 5 were normal in duration and in inducing ovulations in females; but ability to ejaculate sperms was already gone even before April 20 and 28, at least in No. 3, the most quickly activated male.

Histological Conditions

The Testis.—On February 24, after 106 days of 8½ hours duration, the testes of male 4 (Fig. 1) showed seminiferous tubules of about 152 μ diameter, an increase from about 79 μ on December 12 at the autumn quiescent condition (Bissonnette, 1932, Fig. 3) or about 77 μ on November 5 (Allanson, 1932), as compared with about 165–179 μ on February 4 and 8 respectively for the normal ferret (Allanson, 1932). Interstitials were increased in number and activity as compared with those in the completely quiescent autumn condition. Intertubular spaces were distended with lymph, which stained slightly with eosin. Within the tubules were Sertoli cells, spermatogonia, and full-grown spermatocytes; but no further generations of germcells. A lumen was present in the tubules and the syncytial cytoplasm was rather sharply delimited from that of the individual cells. Alto-

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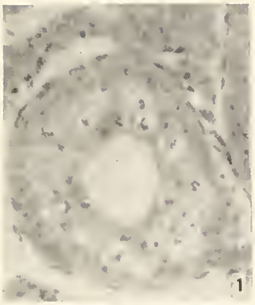
Explanation of Plate I

All figures are photomicrographs taken in the first instance at 335 diameters and reduced to about 190 diameters in reproduction.

Fig. 1. Testis section from "short day" ferret, on 8½ hours of daylight per day from November 10 to February 24—106 days.

Fig. 2. Testis section from "long day" ferret, on normal day-length with 6 hours of electric light added per night, from November 10 to February 24—106 days.

Fig. 3. Testis section from "short day" ferret on March 15—125 days.
Fig. 4. Testis section from "short day" ferret on April 8—149 days.
Fig. 5. Testis section from "long day" ferret on June 8—210 days.
Fig. 6. Testis section from "long day" ferret on June 9—211 days.
Fig. 7. Epididymis section from "short day" ferret, February 24—106 days.
Fig. 8. Epididymis section from "long day" ferret, February 24—106 days.
Fig. 9. Epididymis section from "short day" ferret, March 15—125 days.
Fig. 10. Epididymis section from "short day" ferret, April 8—149 days.
Fig. 11. Epididymis section from "long day" ferret, June 8—210 days.
Fig. 12. Epididymis section from "long day" ferret, June 9—211 days.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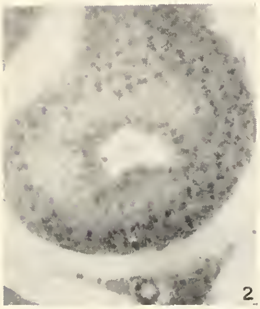
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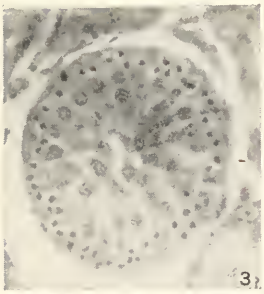
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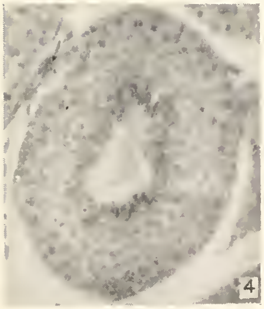
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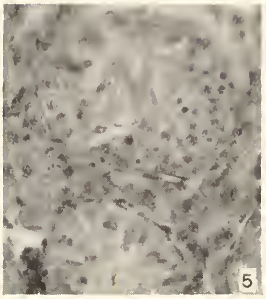
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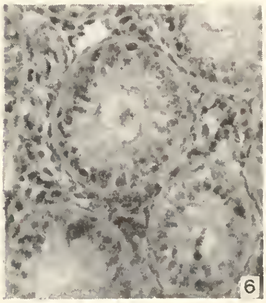
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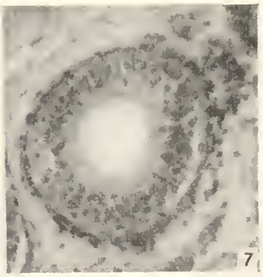
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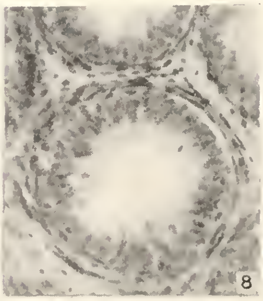
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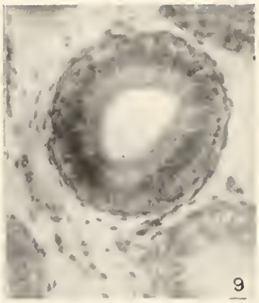
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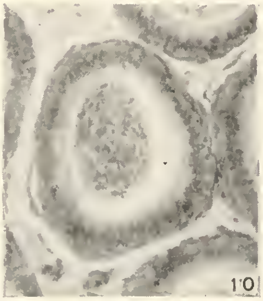
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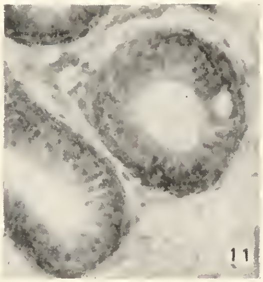
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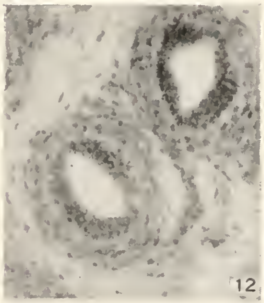
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gether the testes of this animal were definitely retarded in activity by the "short days" as compared with those on the normal light cycle in which spermatogenesis is almost, if not quite, complete by February 4 (Allanson, 1932).

On the same date, after the same period with 6 hours of electric light each night added to normal days, the testes of Male 1 (Fig. 2) were much larger than those just described. Tubule diameters were about 197 μ, even larger than those of ferrets on normal days at March 12 (Allanson, 1932). Interstitial cells were in breeding condition, and intertubular lymph spaces distended with lymph, staining weakly in eosin. Spermatogenesis was complete, and mature sperms were plentiful in the epididymis and vas deferens near the testis (Fig. 8), just as in the animals on normal light cycles on March 2 or 12 (Allanson, 1932).

On March 15th, after 125 "short days," the tubules of the testes of Male 2 (Fig. 3) were about 185 μ in diameter. Interstitials were numerous and active; intertubular lymph spaces large and full. No germ-cells beyond secondary spermatocytes were yet present, except a very few spermatids, not yet showing signs of metamorphosis. Early germ-cell stages were very numerous. This testis had not yet reached the stage normal for February 4 (Allanson, 1932) though its size may not have been retarded.

On April 8, after 149 "short days" the testes of Male 6 (Fig. 4) were in complete reproductive activity in all respects. Sperms were numerous in both testis and epididymis (Fig. 10) and tubules were 176 μ or more in diameter. This male had ejaculated sperms in matings as early as March 7, but not before, so he was also definitely retarded in sexual activation by the "short days." However, the short days did not prevent him from coming into complete sexual activity. Changes in light intensity could account for the activation (Bissonnette, 1933; Marshall and Bowden, 1934).

On June 8, after 210 "long days," the testes of Male 5 (Fig. 5) had passed the climax of activity of germ-cells and were far along in regression. Tubule diameters were only 113–128 μ. However, interstitials were still abundant and apparently active. Lymph spaces were small and not distended. There were no germ-cells beyond synizesis stages of spermatocytes. Lumina were absent in the tubules; cytoplasm was stringy; and many nuclei necrotic. Though this animal was slow to come into activity as compared with the other two "long day" ones, his sex-glands had already long passed their climax and were far into regression, even on a more than usually stimulating light schedule. At this date, ferrets on normal light cycles are still at full activity (Allanson, 1932).

On June 9, after 211 "long days," the testes of Male 3 (Fig. 6), quickest to reach sexual activity, were even farther in regression than those of the preceding animal. Tubule diameters were only 81–99 μ; interstitials were in the typical autumn quiescent condition; and germ-cells reduced to spermatogonia and very few synizesis stages. Only a very few tubules had lumina, and necrotic nuclei were numerous in the stringy cytoplasm.

In both these "long day" animals regression had set in even in spite of a very stimulating schedule of daily light exposures.

The Epididymis (*beside the middle of the testis*).—On February 24, the epididymis of Male 4 (Fig. 7) had reached almost the completely active condition, with epithelial lining cells about 30 μ tall or more, and the cilia-like processes long and abundant. Sperms were not present. The same regions were at the complete breeding condition in Male 1 ("long day") (Fig. 8), with sperms and cellular debris in the lumen tangled in the long cilia-like processes. Tubules were stretched by contents and the epithelial lining was like that in the preceding "short day" ferret.

On March 15, the epididymis of "short day" Male 2 (Fig. 9) was in breeding condition, with epithelial cells about 42 μ tall, but without sperms. On April 8, that of "short day" Male 6 (Fig. 10) was similar but with sperms in the lumen. Lining was stretched until it was only about 30 μ tall.

On June 8, the epididymis of "long day" ferret No. 5 (Fig. 11) was still in breeding condition, with lining cells 32 μ tall and "cilia" abundant. This condition was correlated with active interstitial cells in the testes; but germ-cells far in regression.

On June 9, the epididymis of "long day" ferret No. 3 (Fig. 12), quickest to respond to light stimulation, was far into regression and almost at the autumn quiescent condition. "Cilia" were short and almost indistinguishable; epithelial lining only 15 μ tall or less, and lacking the clear zone next the lumen of the tubule so characteristic of the active condition of these cells.

Both these "long day" ferrets had undergone regression of the epididymis parallelling that of the interstitials of the testis, but lagging behind that of the germ-cell line, more evident in Male 5 than in the more responsive Male 3.

DISCUSSION

The retardation of testis activity in Males 2 and 4 by the shortened days, in spite of increasing daylight intensity, indicates that increasing day-length stimulates or accelerates onset of spring sexual activity or that lack of it holds activity back. The much quicker response of Male 6 may be due to more favorable position of the head and eyes for reception of light when curled up to sleep or to greater individual susceptibility to effects by increasing intensity (Bissonnette, 1932, 1933, and unpublished data; Marshall and Bowden, 1934) than in those more delayed.

The slow reaction of Male 5, on " long days " as contrasted with that of 1 and 3, may be due to greater distance from the bulb or to less favorable reception during sleep; but that could hardly account for its being slower than Male 6. Individual idiosyncrasy appears to be a more likely reason until further study and analysis shed more light on the matter.

In spite of these apparently anomalous exceptions, it appears that, for most young ferrets, an increase in day-length leads to acceleration of testis activity, with interstitials preceding germ-cells in effectiveness; and that libido and accessory sex-organs (epididymis) are closely correlated with interstitial cell changes, in agreement with results from previous studies (Bissonnette, 1932, 1933; Marshall and Bowden, 1934; Hill and Parkes, 1933).

On the other hand, again with some exceptions, a relatively constant, short, winter-like day, throughout the spring season of normally increasing day-length, leads to a delay in onset and a slowing up of the spring activity of the testis and epididymis and a late appearance of sexual libido.

Either type of experimental modification of day-length leads to modification of the sexual cycle in such young males by changing the time of onset of libido and sexual power, as related to ejaculation of sperms and spermatogenesis, from the normal one in nature.

Both behavior and histological studies indicate that, even under increasing stimulating light exposures, greater than normal, sexual regression or " anoestrus " (Hill and Parkes, 1933) sets in after a time. Testis activity and libido (" Oestrus," Hill and Parkes, 1933) may be hastened by these increasing exposures to light, but cannot be maintained indefinitely by them (see Bachman, Collip, and Selye, 1934). Regression comes in spite of them and as the result of changes from within the ferrets, presumably from changes of the anterior hypophysis or immunity to its hormones. This is supported by facts brought out by a preliminary casual study of histological conditions of the hypophyses at autopsy throughout these experiments. These indicate an increased activity of the anterior lobe during the progressive changes of the sex-glands and a falling off during regression. This conclusion has been confirmed by Dr. A. E. Severinghaus, who kindly looked over some of our hypophysis material and agreed with our conclusions. This will be dealt with more fully later.

While regression sets in, in spite of increasing day-lengths, in ferrets, just as in starlings (Bissonnette, 1933, and preceding papers), it is probable that it is hastened by shortening autumn days or that the beginning of the next increase in activity is delayed by the reduction of light. Regression need not depend on a falling light curve but occurs sooner or later even on a rising day-length. How soon regression may be stopped and reversed after it begins or how far destruction of germ-cells must go before the next progressive changes may be induced remains to be seen.

The discovery of Hill and Parkes (1934) that ferrets of unmentioned age, of both sexes, come into "oestrus" in spring, even when subjected to total darkness for 23½ hours or more per day continuously from January 24 onward up to 5½ months, and that a second oestrus may follow after suckling young under these conditions, is very important in this connection. Their conclusion that the increasing length of day in spring is not essential for the appearance of normal oestrus in ferrets in spring, is perhaps too sweeping in its implications and not completely warranted by their published data. "Oestrus," to be normal, must come at the normal time and by the same time sequence as in nature. Their data show that the "oestrus" occurring under their experimental conditions was later than normal and took longer to reach the receptive stage than normal. To be conclusive, their experiment should have begun in November or December, not after their animals had been subject to over a month of increasing light exposures, already known to be sexually stimulating (Bissonnette, 1932, 1933; and their own earlier experiments). This will be discussed more fully in a later paper.

Recent experiments by Allanson, Rowlands, and Parkes (1934), adding light from October 12 onward and following up with injections of pregnancy urine extracts, gave pregnancies from matings on December 29 and January 1, and litters of 9 and 10 young born February 8 and 11 and show that added lighting in autumn increases sexual activity in males as well as females. Some of their males reached complete sexual activity in early January from increased lighting alone. Their litters show that eggs and sperms induced by artificial activation are fertile and viable. Some of our own experiments to be reported later led to complete spermatogenesis and accessory sex-organ activity before November 7, after 36 days of experimental lighting alone. They also showed onset of regression following such activity in spite of increasing light exposures as a result of some internal changes in the animals or on passing the optimum intensity or duration of daily light periods. To date the internal change seems to offer the best explanation, based upon a time factor (Bachman, Collip and Selye, 1934).

SUMMARY AND CONCLUSIONS

1. Three immature male ferrets were kept on " short days " from November 10 till killed for study of testes and epididymides on February 24, March 15, and April 8.

2. Three similar males were kept on " long days " from November 10 until February 24, June 8, and 9.

3. Mating reactions and sperm ejaculating power were tested from January 13 onward.

4. Onset of sexual activity was delayed considerably in two of the " short day " animals, as compared with normals, only slightly in the third.

5. It was hastened in two " long day " animals, not in the third, which was late.

6. In both groups, interstitials came into activity before germ-cells, and, in the " long day " animals, remained active longer than germ-cells, when regression followed the climax of activity.

7. Changes in epididymides were more closely correlated with those of interstitials than with those of germ-cells.

8. In " long day " ferrets, quickly activated or not, regression set in well before June 8, at which time males on normal light cycles are at complete sexual activity, in spite of increasing duration and intensity of daily light exposures.

9. Therefore, while sexual activity may be induced or hastened by increasing daily light in young male ferrets, as in females, in these males regression sets in after a time even in spite of increasing light. Regression in such males is related either to changes within the animals, like fatigue or immunity reactions, or to an increase of lighting above the optimum or to both. It is possible that, in nature, the shortening days after June 21 hasten it or intensify its effects.

BIBLIOGRAPHY

ALLANSON, M., 1932. The Reproductive Processes of Certain Mammals. III. The Reproductive Cycle of the Male Ferret. *Proc. Roy Soc.*, Ser. B, **110**: 295.

ALLANSON, M., I. W. ROWLANDS, AND A. S. PARKES, 1934. Induction of Fertility and Pregnancy in the Anoestrous Ferret. *Proc. Roy. Soc.*, Ser. B, **115**: 410.

BACHMAN, C., J. B. COLLIP, AND H. SELYE, 1934. Anti-Gonadotropic Substances. *Proc. Soc. Exper. Biol. Med.*, **32**: 544.

Bissonnette, T. H., 1932. Modification of Mammalian Sexual Cycles. Reactions of Ferrets (Putorius vulgaris) of Both Sexes to Electric Light Added after Dark in November and December. *Proc. Roy. Soc.*, Ser. B, **110:** 322.

Bissonnette, T. H., 1933. Light and Sexual Cycles in Starlings and Ferrets. *Quart. Rev. Biol.*, **8:** 201.

Hill, M., and A. S. Parkes, 1933. Studies on the Hypophysectomized Ferret. IV. Comparison of the Reproductive Organs during Anoestrus and after Hypophysectomy. V. Effect of Hypophysectomy on the Response of the Female Ferret to Additional Illumination during Anoestrus. VI. Comparison of the Response to Oestrin of Anoestrous, Ovariectomized and Hypophysectomized Ferrets. *Proc. Roy. Soc.*, Ser. B, **113:** 530.

Hill, M., and A. S. Parkes, 1934. Effect of Absence of Light on the Breeding Season of the Ferret. *Proc. Roy. Soc.*, Ser. B, **115:** 14.

Marshall, F. H. A., and F. P. Bowden, 1934. The Effect of Irradiation with Different Wave-lengths on the Oestrous Cycle of the Ferret, with Remarks on the Factors Controlling Sexual Periodicity. *Jour. Exper. Biol.*, **11:** 409.

FURTHER ANALYSIS OF THE PROTECTIVE VALUE OF BIOLOGICALLY CONDITIONED FRESH WATER FOR THE MARINE TURBELLARIAN, PROCERODES WHEATLANDI. IV. THE EFFECT OF CALCIUM

R. B. OESTING AND W. C. ALLEE

(*From the Marine Biological Laboratory and the University of Chicago*)

In a series of studies (1928, 1929, 1933) Allee has shown that the marine turbellarian, *Procerodes wheatlandi,* resists cytolysis longer in extremely hypotonic water, other conditions being equal, if such water has been biologically conditioned than if it lacks biological contamination. These relations hold even though the total concentration of electrolytes, as measured by the conductivity method, is the same. The balance of electrolytes is necessary in such experimental tests, since an increase in their concentration in the proportions found in sea water likewise confers protection.

Protective biological conditioning results when other *Procerodes* have previously been exposed to the fresh water, and especially so if some few *Procerodes* have recently died and disintegrated in it. The protective action of this biologically conditioned fresh water is maintained after dialysis to the same conductance as that of extremely dilute sea water controls. Water extracts of marine amphipods and of fresh water planarians, *Euplanaria novangliæ* (=*Planaria maculata*), show these same effects, as does water from cultures of the latter and water from sterilized hay infusions of a practically pure culture of *Paramecium.* The protective effects are not due to pH differences, or to a depressing action such as is produced by exposure to dilute alcohol or to ethyl urethane. The protective agent is not adsorbed by activated charcoal, or by coagulated egg white.

The onset of cytolysis was determined in these experiments by inspection with a hand lens. The results have been confirmed by histological studies on worms taken from Allee's 1933 experiments (Fowler, 1935).

Pantin (1931*a* and *b*) and Weil and Pantin (1931) have studied the adaptation of *Procerodes* (=*Gunda*) *ulvæ* to fresh waters. Their results bear on our problem since the two species are closely related taxonomically and in habitat toleration. *P. ulvæ* invades small streams where conditions are suitable; it may extend up these to, or slightly above, mean high water at neap tides (Ritchie, 1934). The reaction of this planarian in fresh waters varies with the chemical composition of the water. The worms swell less and recover more completely on their return to sea water if placed in fresh waters rich in calcium rather than in calcium-deficient waters. In nature, Ritchie (1934) has found them living in fresh water with as little as 5 mgm. per liter of calcium for as long as five days when periods of calm weather, with the resulting lack of salt splash, coincide with neap tides.

Materials and Methods

P. wheatlandi were collected daily from the same locality as in the previous studies of Allee. *Euplanaria novangliæ* were collected from a fresh water pond known to be low in salt content; the pond water used came from the same pond which was also the source of these planarians and of pond water in Allee's latest work (1933).

The interval before the beginning of cytolysis in hypotonic waters depends on several factors, among which are the following: Small worms begin and undergo cytolysis more rapidly than do larger ones; worms long in the laboratory are less resistant than are those freshly collected; the more often the medium is renewed, the sooner cytolysis begins. The higher the temperature, or the lower the specific conductance of the water, the less the resistance of *Procerodes* (cf. Allee, 1933).

Assays of the protective value of various media were made, keeping these factors as nearly identical as possible for the worms whose resistance was being comparatively tested except that variations in specific conductance were used as a tool in the analyses. The worms were first isolated in a small drop of sea water on the curved bottom of the salt cellar type of watch glass; they were then washed five times with the respective media into which they were to be placed. The worms were paired for comparative assays before the test began; the members of each pair were selected for similarity in size, activity, and laboratory age. After the washings, 2 cc. of the respective media were run over each worm; this liquid was renewed every one, two, or four hours, depending on the severity of the media and the laboratory age of the worms. The survivals were all tested at room temperature in a room with north exposure which was not subjected to rapid changes in temperature. The highest temperature recorded for the summer in this room was 25.5° C. Examinations were made every fifteen minutes with a ten-power hand lens until the beginning of fatal cytolysis was noted. Ten worms were isolated into each type of media being tested in an assay.

Conductivity measurements were made using a No. 7651 Leeds and Northrup potentiometer and a Washburn type conductivity cell designed for solutions with low conductance. Specific conductances are given in mhos $\times 10^5$. Allee has previously given conductivity measurements in terms of ohms resistance. His data may be compared with those given in the present report by the use of the following data: under the conditions given, 6,000 ohms $= 5.42 \times 10^{-5}$ mhos; 2,500 ohms $= 13.05 \times 10^{-5}$ mhos and 1,700 ohms $= 19.20 \times 10^{-5}$ mhos.

Calcium analyses were made using the Van Slyke and Sendroy (1929) method. The accuracy of this method to within the one per cent claimed for it was confirmed by tests on the recovery of calcium added to distilled water and to the other media used in this work.

TABLE I

The Protective Action of Calcium for Procerodes Isolated into Extremely Hypotonic Water

Experiment No.	Medium	Calcium added		Calcium not added		Difference in hours
		Mhos $\times 10^5$	Hours survived	Hours survived	Mhos $\times 10^5$	
5	Tap	8.38	2.74	1.80	7.30	0.94
6	Sea	30.12	2.23	1.25	28.69	0.98
6*a*	Tap	8.83	3.95	2.43	7.15	1.52
8	Tap	21.29	9.33	4.00	7.07	5.33
8*a*	Sea	40.80	9.31	4.03	26.53	5.28
9	Pond	5.02	7.20	3.30	4.79	3.90
10	Pond	5.50	5.50	3.55	4.79	1.95
10*a*	Pond	6.27	15.80	5.32	4.56	10.48
11	Pond	7.43	3.23	2.14	4.79	1.09
12	Pond	7.13	2.63	2.20	4.79	0.43
13	Pond	5.40	4.34	3.00	4.79	1.34
21	Pond	6.25	5.98	4.08	4.79	1.90
Averages			6.02	3.09		2.92

Statistical probability 0.005

EXPERIMENTAL RESULTS

Calcium, as $CaCl_2$, was added to tap, pond and to extremely hypotonic sea water in a group of twelve experiments with results which are summarized in Table I. In each assay, the worms survived longer, on the average, in the water to which calcium had been added than did control worms in otherwise similar water. The amount of calcium used differed in different tests; in all cases the increase in specific conductance is a measure of the calcium added. Further comparisons can be made from the fact that in Experiment 8, 1 cc. of M/10 $CaCl_2$ was added to 200 cc. of tap water and in Experiment 8*a* the same amount was added to 200 cc. of 1 : 300 sea water.

Although varying amounts of calcium were added and further variations were caused by differences in laboratory age and in the number of changes of the media during the assays, when the data given in Table I are considered as twelve paired experiments, the difference in survival of 2.92 hours has a statistical probability of 0.005.[1] The coefficient of correlation between the differences in the amount of calcium as measured by specific conductance and the differences in the resistance of the worms is 0.3691 ; this is statistically significant.

TABLE II

Relative survival of Procerodes isolated in tap water and in dilute sea water. Time is given in hours and each value is the average for 10 worms; specific conductance is in mhos.

Experiment No.	Dilute sea water		Tap water	
	Survival	Sp. cond. × 10^5	Survival	Sp. cond. × 10^5
1	4.825	19.07	5.725	6.64
2	6.667	20.14	8.566	6.38
3	4.697	25.90	7.865	6.79
4	2.768	28.69	2.100	7.82
5	1.535	27.95	1.800	7.30
6	1.250	28.69	2.425	7.15
7	7.875	39.75	8.150	7.07
8	4.775	26.53	4.000	7.07
Averages	4.299	27.09	5.079	7.03

The relatively greater protection from hypotonic water furnished by calcium in comparison with that given by other electrolytes in the proportions found in sea water is illustrated by the data summarized in Experiments 8 and 8*a*, Table I, and the further data given in Table II. In Experiments 8 and 8*a*, despite the differences in conductance, there is no significant difference in survival of worms in tap water or in 1 : 300 sea water and again there is no significant difference in survival in these two waters after the same amount of calcium is added. There is a decidedly significant difference between the survival of the worms in either water before and after the addition of calcium.

The waterworks of Falmouth, which supplies the tap water used in these experiments, now uses a lime treatment which materially increases the calcium content. This treatment was started since Allee's last preceding experiments. The presence of this calcium probably accounts for the markedly greater protection of *Procerodes* in this water over that which would be expected from its low specific conductance. The dilutions of sea water contained approximately four times as much electrolytes as did the tap water; rough calculations of the calcium content from analyses furnished by the Falmouth waterworks showed this to be practically the same in the two. The survival of the *Procerodes* isolated in the two types of water was approximately the same in both; the mean difference of 0.78 hours longer survival in tap water lacks statistical significance (P. = 0.133).

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[1] Values of 0.05 or less are usually considered statistically significant. "Student's" method of paired comparisons was used in all statistical analyses.

TABLE III

Survival of Procerodes in planarian-conditioned fresh water as compared with that shown in different control media. Ten worms were assayed in each experiment except No. 13 in which eight were used.

Experiment No.	Hours of survival			
	I Planarian-conditioned water	II Pond water	III Pond water + $CaCl_2$	IV Pond water + sea water
9	4.025	3.300	7.900	—
10	3.925	3.550	4.650	—
11	3.650	2.650	3.725	2.150
12	2.525	2.275	2.725	2.200
13	4.156	2.594	4.219	3.188
20	1.525	0.750	1.350	1.225
21	5.250	2.925	5.975	4.075
Means	3.579	2.578	4.363	2.568

Statistical analysis

	Difference		Probability	Cases
I–II	0.9900		0.0000	68
III–I	0.9375		0.0004	68
III–II	1.7941		0.0000	68
I–IV	0.8490		0.0010	48
IV–II	0.3125		0.2508	48
III–IV	1.0313		0.0000	48

It is evident from these data that calcium delays the onset of cytolysis when *Procerodes* are isolated in extremely hypotonic fresh waters. Any factor which would tend to increase the amount of calcium present then, should confer such protection. This leads to the fundamental question of the present inquiry: Is the protective value of biologically conditioned water to be attributed to a differential increase in calcium which accompanies conditioning? It is at once obvious that the addition of sea water to a control sample of fresh water until its total electrolyte content equals that of a biologically conditioned sample as measured by the conductivity method, while furnishing a basis for an adequate check on protection due to increased osmotic pressure, gives inadequate control over any specific electrolyte such as calcium. It is necessary to test directly for the amount of calcium present and to confirm its effectiveness in biologically conditioned water.

Unless otherwise stated, the amount of biological conditioning used was that furnished by 200 *Euplanaria novangliæ* living in one liter of fresh pond water for approximately 44 hours, or its equivalent obtained by using fewer worms for a longer period. Sea water and calcium chloride were added respectively to other portions of fresh pond water until the specific conductance of all three solutions was the same. As an additional control, except in Experiments 9 and 10, a sample of untreated pond water was assayed together with the above media. The average survival times of *Procerodes* from seven such experiments are shown in Table III.

TABLE IV

Calcium analyses of assayed samples of media from Table III; results shown in mgm. calcium per liter.

	I Planarian-conditioned water	II Pond water	III Pond water + $CaCl_2$	IV Pond water + sea water
Calcium content......	2.072	0.809	3.410	1.018

These data, together with the statistical analyses, show that planarian-conditioned water has a protective value lying between that of pond water plus sea water and pond water plus calcium chloride when all are of the same electrolytic content. When, however, the amount of calcium present in the planarian culture water (Table IV) was determined and calcium chloride was added to pond water to bring the calcium content to that of the planarian-conditioned water, no difference could be detected between the effectiveness of these two waters in protecting the *Procerodes* isolated in them. These results are summarized, together with a statistical analysis, in Table V.

To determine whether any factor other than calcium might be involved in the protection furnished by planarian-conditioning, a medium

TABLE V

Procerodes survival in pond water and planarian-conditioned pond water with the same calcium content.

Experiment No.	Hours of survival			
	I Planarian-conditioned water	II Pond water	III Pond water + sea water	IV Pond water + $CaCl_2$
26	5.250	5.650	3.850	8.300
27	1.875	2.150	1.150	3.500
Means	3.563	3.900	2.500	5.900

Statistical analysis

	Difference	Probability	Cases
I– II	−0.3375	1.0000	20
I–III	1.0625	0.0396	20
IV– I	2.3375	0.0052	20
II–III	1.4000	0.0230	20
IV– II	2.0000	0.0344	20
IV–III	3.4000	0.0000	20

was made up to represent a synthetic river water (Henderson, 1913, p. 113). To each liter of water the following salts were added:

100 mgm. $CaCl_2$
50 mgm. $MgSO_4$
25 mgm. NaCl
10 mgm. KCl

This water in full, half- and quarter-strengths, when conditioned by planarians, showed no change in the total electrolytic content; likewise there was no protection for *Procerodes* when compared by the usual assay to the survival shown in similarly treated but unconditioned synthetic river water. The results of these experiments together with a statistical analysis are summarized in Table VI. From all this evidence, we can safely conclude that an increase in calcium is the factor furnishing the protection to *Procerodes* in planarian-conditioned pond water.

Allee's previous work had shown that water extracts of *Procerodes* themselves furnished a potent protection for other worms of the same species isolated in extremely hypotonic media. The relation between this protection and the calcium content of the water was also tested.

TABLE VI

A summary of tests which indicate that the protection furnished by calcium is sufficient to account for the protective value of planarian-conditioned water. Each of the survival times given is the average for ten worms.

Experiment number	Hours survival		Amount of conditioning	Laboratory age of *Procerodes*	Mhos × 10^5	Media strength	Media changed	Difference
	Planaria culture	Control						
14	10.225	5.625	100 worms in 250 cc. 16 hrs.	1 day	48.70	Full	Every hour	+4.60
15	6.550	6.800	100 worms in 500 cc. 44 hrs.	2 days	48.70	Full	Every hour	−0.25
16	21.75	19.30	14 worms in 250 cc. 68 hrs.	over 5 days	48.70	Full	Every hour*	+2.45
17	5.65	6.30	200 worms in 500 cc. 20 hrs.	3 days	26.15	Half	Every 2 hours	−0.65
18	6.95	7.15	100 worms in 500 cc. 44 hrs.	4 days	26.15	Half	Every 2 hours	−0.20
19	7.00	6.20	60 worms in 500 cc. 68 hrs.	4 days	26.15	Half	Every 2 hours	+0.80
23	5.50	6.90	200 worms in 500 cc. 20 hrs.	1 day	12.32	Quarter	Every 2 hours	−1.90
24	8.90	9.35	100 worms in 500 cc. 44 hrs.	2 days	12.32	Quarter	Every 2 hours	−0.45

Average . . . . +0.55
Statistical Probability 0.4500

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* Media exhausted after six changes.

Procerodes extract was prepared as in the earlier work. After five washings with distilled water, 500 *Procerodes* were boiled in distilled water for about 15 minutes. The beaker was then covered and set aside for 24 hours. Controls were made up to the same specific conductance as the extract by adding calcium chloride to distilled water and sea water to distilled water respectively. All three media were then assayed for their protective value to *Procerodes* and were then analyzed for calcium. It is evident from an examination of Tables VII and VIII which summarize these data that calcium is also the factor in *Procerodes* extracts which furnishes the observed protection for *Procerodes*. Since making a water extract of *Procerodes* is a short-cut method for preparing *Procerodes*-conditioned water, it is unnecessary to examine this possibility for further confirmatory evidence that an increase in calcium is the fac-

TABLE VII

The protective value of *Procerodes* extracts for *Procerodes* isolated in hypotonic media; each survival time given is the average for ten worms.

Experiment No.	Hours survival		
	I *Procerodes* extract	II Dist. H_2O + $CaCl_2$	III Dist. H_2O + sea water
28	6.150	6.350	4.150
29	2.100	3.000	1.750
Means	4.125	4.675	2.950
	Statistical analysis		
	Difference	Probability	Cases
I–II	0.550	0.5280	20
I–III	1.175	0.0344	20
II–III	1.725	0.0050	20

TABLE VIII

Calcium analyses of assayed samples from Table VII; results shown in mgm. per liter.

	Procerodes extract	Dist. H_2O + $CaCl_2$	Dist. H_2O + sea water
Calcium content	7.20	8.66	0.42

tor furnishing protection to *Procerodes* in biologically conditioned fresh waters.

Discussion

These experiments were carried on by essentially the same methods used by Allee in his preceding studies and support his results wherever similar points were being tested. Specifically, in addition to more general relations, these experiments confirm the earlier findings, that, other conditions being equal, *Procerodes* survive longer (1) if the osmotic pressure is increased by the addition of sea water; (2) if the hypotonic water is biologically conditioned (*a*) by the presence of living fresh water planarians or (*b*) by the presence of water extracts of freshly killed *Procerodes.*

In addition, the earlier results are extended by the demonstration that there is more calcium added than would be expected from its proportionate concentration in sea water and that calcium has protective value greater than would be expected from its osmotic effect. There is nothing in the earlier experiments which is out of harmony with the present findings.

In certain of Allee's work (1929, 1933) the conditioned water was dialyzed to bring the specific conductance to the desired experimental level. Such dialyzed solutions were definitely protective. This protection can be explained adequately in the light of the present work if one considers the amounts of calcium introduced into conditioned water, for example, by *Procerodes* extracts (cf. Table VIII). Even if one assumes that the collodion membranes used were freely permeable to calcium, the calcium content could be reduced by more extreme dialysis than that used and still leave enough calcium to be definitely protective as compared with a similarly hypotonic sea water control.

The demonstration that the protective action of these biologically conditioned solutions is due to the increased calcium content in so far as the resistance of *Procerodes wheatlandi* to hypotonic water is concerned, brings this phenomenon into line with the greater resistance shown by *Procerodes* (= *Gunda*) *ulvæ* to fresh water when the calcium content is high. This is hardly the place, nor is *Procerodes* necessarily the most favorable material for an inquiry into the mechanism of calcium protection under these conditions.

The analyses of Pantin and his associates and of Beadle (1931, 1934) indicate that *P. ulvæ* lives in osmotic equilibrium with sea water and reaches a steady physiological state in certain fresh waters. This steady state is not the result of simple osmotic balance but is dependent largely on the calcium content of the water. It is maintained in part by a change in the permeability of the surface epithelium and is accompanied by increased respiration; it cannot be maintained indefinitely in extremely hypotonic waters. It seems highly probable (cf. Weil and Pantin, 1931) that the mechanism with these planarians is related to that in other cells in certain of which it has been shown, *Arbacia* eggs for example (McCutcheon and Lucke, 1928), that calcium decreases cell permeability to water and (Heilbrunn, 1930) favors membrane formation at a broken surface. The protection in these cases appears to be due to a decreased water intake rather than to a decreased leaching out of materials essential for continued existence.

We have not investigated the range of concentrations of calcium which would delay cytolysis in *Procerodes* placed in hypotonic water; in fact, we have established neither the upper nor the lower effective concentration. We know that an increase in specific conductance produced by the addition of calcium chloride equal to 1.08×10^{-5} mhos will give measurable protection. A rough calculation shows that this is approximately equivalent to the introduction of M/2600 $CaCl_2$. This approaches the lower limit of effectiveness under the conditions of our experiments. Buchanan (1935) reports that complete cytolysis readily occurs in *Euplanaria dorotocephala* in distilled water while in distilled water solutions of $CaCl_2$ there is no cytolysis in concentrations from M/500 to M/40,000 and cytolysis is distinctly delayed in a dilution of M/100,000. He found a detectable protective action in a M/1,000,000 solution.

We have been engaged in investigating the relation between calcium and the protective action of biological conditioning in extremely hypotonic waters when the calcium content is relatively low. We have little evidence regarding the effectiveness or non-effectiveness of such conditioning when the calcium is high. There are some indications in the first three significant lines in Table VI where full strength synthetic river water was used, that such conditioning may be effective. If so, some other mechanism than an increase in calcium is probably acting. These conditions approximate those found when a hard water stream from a limestone region flows into the ocean (cf. Breder, 1934). There is evolutionary and ecological as well as physiological interest in the relations between numbers present and the effectiveness of the invasion of such waters. Our present work does not deal with this question. However, it does show that marine invasions by animals with the physiological requirements of *Procerodes* into such relatively soft fresh waters as we have investigated is definitely favored by the presence in these waters of calcium excretors such as *Euplanaria novangliæ* has been shown to be.

We have no information concerning the actual importance of such conditioning under natural conditions.

Throughout these studies there has been evidence of two opposing effects of numbers upon the resistance of these worms to fresh water: (*a*) the protective effect of freshly conditioned water or of numbers of animals present and (*b*) the harmful effect of numbers which shows up in conditioned media that have been allowed to go stale. At the osmotic level of these experiments, the protective effect is due to an increase in calcium and while the harmful effect has not been analyzed, it is a good *a priori* guess that the observed ill effects are associated with the accumulation of waste products of metabolism or to decomposition products of these.

The series of experiments of which this is the fourth report, were begun and have been prosecuted primarily in an investigation of a phase of mass physiology associated with animal aggregations (cf. Allee, 1931, 1934). The original impetus towards these particular experiments came from the observations of Drzewina and Bohn (1920, 1928) that the marine turbellarian *Convoluta roscoffensis* survives longer in hypotonic water if present in numbers than if isolated. This protection they attributed to the more rapid production of some sort of autoprotective substance by the group than would be possible for a single individual. This suggestion is now seen to have been essentially correct and, under the conditions of our experiments, to be composed of nothing more mysterious than calcium.

SUMMARY

1. An increase in the calcium content of extremely hypotonic water, when the calcium content is less than that found in sea water, delays the onset of fatal cytolysis for *Procerodes wheatlandi* isolated in such media.

2. Other electrolytes, even when increased appreciably, do not show this same protection; however, some protection of an osmotic nature is apparent.

3. The increase of calcium in biologically conditioned fresh waters is adequate to explain their observed protection for *Procerodes.*

LITERATURE CITATIONS

ALLEE, W. C., 1928. Studies in Animal Aggregations: Mass protection from fresh water for Procerodes, a marine turbellarian. *Jour. Exper. Zool.,* **50**: 295.

ALLEE, W. C., 1929. Studies in Animal Aggregations: Mass protection from hypotonic sea-water for Procerodes, a marine turbellarian, with total electrolytes controlled. *Jour. Exper. Zool.,* **54**: 349.

ALLEE, W. C., 1931. Animal Aggregations: A Study in General Sociology. University of Chicago Press.

ALLEE, W. C., 1933. Studies in Animal Aggregations: Further analysis of the protective value of biologically conditioned fresh water for the marine turbellarian, Procerodes. *Physiol. Zool.*, **6**: 1.

ALLEE, W. C., 1934. Recent Studies in Mass Physiology. *Biol. Rev.*, **9**: 1.

BEADLE, L. C., 1931. The Effect of Salinity Changes on the Water Content and Respiration of Marine Invertebrates. *Jour. Exper. Biol.*, **8**: 211.

BEADLE, L. C., 1934. Osmotic Regulation in Gunda ulvæ. *Jour. Exper. Biol.*, **11**: 382.

BOHN, G., AND A. DRZEWINA, 1920. Variations de la sensibilité à l'eau douce des Convoluta, suivant les états physiologiques et le nombre des animaux en expérience. *Compt. Rend. Acad. Sci.*, **171**: 1023.

BREDER, C. M., JR., 1934. Ecology of an Oceanic Fresh-Water Lake, Andros Island, Bahamas, with Special Reference to its Fishes. *Zoologica*, **18**: 57.

BUCHANAN, J. W., 1935. An Analysis of Physiological States Responsible for Antero-posterior Disintegration in Planaria dorotocephala. *Biol. Gen.*, in press.

DRZEWINA, A., AND G. BOHN, 1928. Les Convoluta. *Ann. Sci. Nat. Zool.*, *X.*, Ser. **11**: 299.

FISHER, R. A., 1925. Expansion of "Students'" Integral in Powers of N^{-1}. *Metron*, **5**: 109.

FOWLER, LILLIAN HENDERSON, 1935. Relative Effect of Biologically Conditioned and Other Extremely Hypotonic Water on the Tissues of the Marine Turbellarian, Procerodes wheatlandi. *Physiol. Zool.*, **8**: No. 2.

HEILBRUNN, L. V., 1930. The Action of Various Salts on the First Stage of the Surface Precipitation Reaction in Arbacia Egg Protoplasm. *Protoplasma*, **11**: 558.

HENDERSON, L. J., 1913. The Fitness of the Environment. Macmillan.

MCCUTCHEON, M., AND B. LUCKÉ, 1928. The Effect of Certain Electrolytes and Non-electrolytes on Permeability of Living Cells to Water. *Jour. Gen. Physiol.*, **12**: 129.

PANTIN, C. F. A., 1931*a*. The Adaptation of Gunda ulvæ to Salinity: I. The environment. *Jour. Exper. Biol.*, **8**: 63.

PANTIN, C. F. A., 1931*b*. The Adaptation of Gunda ulva to Salinity: III. The electrolytic exchange. *Jour. Exper. Biol.*, **8**: 82.

RITCHIE, A. D., 1934. Habitat of Procerodes ulvæ. *Jour. Mar. Biol. Ass.*, **19**: 663.

"STUDENT," 1925. New Tables for Testing the Significance of Observations. *Metron*, **5**: 105.

VAN SLYKE, D. D., AND J. SENDROY, JR., 1929. Gasometric Determination of Oxalic Acid and Calcium, and its Application to Serum Analysis. *Jour. Biol. Chem.*, **84**: 217.

WEIL, E., AND C. F. A. PANTIN, 1931. The Adaptation of Gunda ulvæ to salinity. II. The water exchange. *Jour. Exper. Biol.*, **8**: 73.

ON THE INFLUENCE OF PYOCYANINE ON THE RESPIRATION OF THE SEA URCHIN EGG

JOHN RUNNSTRÖM[1]

(From the Laboratories of the Rockefeller Institute for Medical Research, New York, and the Marine Biological Laboratory, Woods Hole, Mass.)

Barron (1929) has demonstrated that the respiration of the unfertilized *Arbacia* and *Asterias* egg is increased by methylene blue. Runnström (1930) reported similar observations for the *Paracentrotus* egg. Runnström (1930, 1932) and Örström (1932) studied chiefly the influence of dimethylparaphenylene diamine on *Paracentrotus eggs.* This compound penetrates the eggs and causes a considerable increase of respiration both of the fertilized and the unfertilized eggs. The respiration of the eggs in the diamine solution is inhibited by cyanide and carbon monoxide. The degree of inhibition by cyanide is the same in the unfertilized and fertilized diamine eggs. The same holds true for the inhibition by CO, if the respiration is equal in the unfertilized and fertilized diamine eggs. The degree of inhibition by CO is dependent on the rate of reduction of the iron-containing enzyme of Warburg (1932). The higher the rate of reduction the more accessible is the enzyme to CO, which reacts with the reduced form of the enzyme (Warburg). Runnström inferred from his inhibition experiments that dimethylparaphenylene diamine is not auto-oxidized in the sea urchin egg but must be oxidized by the reduction of the iron-containing enzyme. Further, it was inferred that this enzyme is also present in the unfertilized egg in a reactive state, but the rate of oxidation is limited by a block in the chain of "carriers" (Keilin, 1929) which connects the iron-containing enzyme and the substrate-dehydrase system.

Barron did not find any inhibition of the respiration enhanced by methylene blue on addition of cyanide. This increase of the respiration is not due to a higher activity of the iron-containing enzyme. Methylene blue is reduced by systems in the cell which cannot react directly with molecular oxygen. It seemed to the writer of interest to try the action of pyocyanine on the respiration of the sea urchin egg. Pyocyanine belongs with respect to its oxidation reduction potential to the same range as methylene blue. As shown by Friedheim and Michaelis (1931) and further by Michaelis, Hill, and Schubert (1932), the pyocyanine can be oxidized or reduced in two steps, each step involving the transfer of one electron. Methylene blue, on the other hand, is oxidized or reduced in one step, each step involving the transfer of two electrons. This difference may be physiologically significant. Friedheim (1931) has shown that pyocyanine is a more effective catalyzer of some oxidations than methylene blue. Further (Friedheim, 1934), he found that the aerobic glycolysis of tumors can be decreased in presence of pyocyanine. The present writer and Michaelis (Runnström and Michaelis, 1934) have proved that the action of methylene blue and pyocyanine is different in the system hemolyzed red blood cells plus hexosephosphate. The oxygen uptake is the same with both dyestuffs, but in the presence of pyocyanine a coupling between the respiration and the synthesis of phosphate esters is induced, which does not exist or is much less conspicuous in the same system containing methylene blue.

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[1] Fellow of the Rockefeller Foundation.

Table I

Unfertilized Eggs	Control	Pyocyanine			
		0.006%	0.009%	0.012%	0.015%
cu. mm. O_2 in 150 min	30	75	84.5	93	93
Increase, *per cent*		125	180	210	210
Fertilized Eggs					
cu. mm. O_2 in 150 min	112	128	204	212	220
Increase, *per cent*		14	80	90	96

Suspension: 4.5 per cent.

The oxygen consumption was measured in the present research by Warburg's manometric method. The type of vessels used was that described by Borei (1934). It has proved to be useful for experiments with the sea urchin eggs. Into each vessel always 3 cu. cm. of the egg suspension was introduced. A 5 per cent solution of KOH was introduced into the inset as well as into one of the two side arms. The concentration of the suspension was determined by centrifuging in capillary tubes as described by Runnström (1933). The values obtained were corrected for the interstitial water by multiplication with the factor 0.64, cf. Teissier (1929). All conclusions are based on comparisons between values obtained in experiments with uniform material. Thus the exact absolute concentration of the suspensions is of minor importance.

Table I gives the evidence that the respiration is increased considerably in both fertilized and unfertilized eggs on addition of pyocyanine. The percentage increase is much higher in the unfertilized than in the fertilized eggs. On the other hand, the absolute values of the oxygen consumption in the unfertilized eggs are lower than those of the fertilized eggs. The highest value obtained in the unfertilized eggs is still somewhat below that found in the normal fertilized eggs. The eggs can be fertilized in the solution of pyocyanine in sea water. As can be predicted from Table I, the percentage increase of the oxygen consumption is much lower than under normal conditions. At the concentration 0.006 per cent pyocyanine the increase is only 75–125 per cent, while in the control the increase amounts to 400–500 per cent. The eggs treated with pyocyanine were transferred at the end of the measurements to normal sea water. The unfertilized eggs are in no way activated and could be fertilized. The division of the fertilized eggs took place in the pyocyanine solution. In the higher concentrations (0.012–0.015 per cent) the division was, however, delayed or suppressed in spite of the increased respiration. The eggs developed after transfer to normal sea water to the pluteus stage. A certain injurious effect of the higher concentrations of pyocyanine was evident, even after transfer to normal sea water.

TABLE II

	Unfertilized Eggs					
Pyocyanine, *per cent*	0	0.003	0.006	0.02	0.02	0.02
HCN n	—	—	—	—	0.001	0.0025
cu. mm. O_2 in 120 min	24	36	64	82	110	128
Increase, *per cent*	—	49	167	240	360	435

An *increase* of the oxygen consumption was regularly observed on addition of HCN to the unfertilized eggs in pyocyanine.

In the highest HCN concentration the oxygen consumption was equal to or somewhat higher than in the fertilized control eggs.

The same material was used for the experiments on which Tables II and III are based. The figures are thus directly comparable. The respiration of the fertilized pyocyanine eggs is decreased by addition of HCN. The residual respiration is about 70 per cent. From other experiments the residual respiration in presence of 0.0025 N HCN is known to be only about 30–35 per cent. It is easy to see that the part of the respiration induced by pyocyanine, $266 - 123 = 140$ cu. mm., is not inhibited by HCN: 30 per cent of 123 is about 40 cu. mm.; $140 + 40$ cu. mm. is equal to 180 cu. mm., which is the oxygen consumption observed in the eggs in pyocyanine plus HCN. In some cases the residual respiration was somewhat higher than expected according to the above calculation but there is always an inhibition.

No data are available about the HCN inhibition in the unfertilized eggs of *Arbacia* in sea water without pyocyanine, but to judge from figures (Runnström, 1930) valid for the *Paracentrotus* egg, the inhibition is weak in the normal (not aged) unfertilized egg as compared with the inhibition in the fertilized egg.

The rise of the oxygen consumption of the eggs in sea water plus pyocyanine is greater than on addition of methylene blue. In one experiment the increase of respiration in unfertilized eggs was 60 per cent in presence of 0.006 per cent methylene blue, while the increase caused by 0.006 per cent pyocyanine (a concentration very nearly equimolecular to that of the methylene blue in this experiment) was 200 per cent.

At the suggestion of Dr. Alfred Mirsky, the eggs were frozen by means of solid CO_2 and ether at a temperature of about —80° C. These eggs were then allowed to thaw slowly at room temperature. The thawing breaks up the eggs. A part of the egg contents is dissolved in the sea water, another part remains undissolved. The thawed eggs show a certain oxygen uptake, but this is considerably enhanced by the addition of pyocyanine or methylene blue. In many experiments hexose phosphate was added, which increased the oxygen uptake 100–200 per cent. (Table IV.)

Table III

	Fertilized Eggs		
Pyocyanine, *per cent*	0	0.02	0.02
HCN n	—	—	0.0025
cu. mm. O_2 in 120 min	123	266	181

Suspension: 4 per cent.

Pyocyanine gives a somewhat higher oxygen consumption than methylene blue added to the above system with hexose phosphate. In one experiment the oxygen consumption in presence of methylene blue was 97 cu. mm., while in presence of pyocyanine it was 123 cu. mm. in 120 minutes.

In the experiments cited above (Table III) it is evident that the respiration in fertilized eggs to which HCN has been added is increased by addition of pyocyanine to a level above the normal. The concentration of HCN in question blocks the mitotic process. If the eggs were brought into the HCN sea water soon after fertilization, the division stopped in an early prophase, in the stage characterized in the living material by the appearance of the clear streak near to the nucleus. A certain increase of the volume of the nucleus takes place but the dissolution of the nuclear membrane does not occur in the presence of HCN. The block is due without any doubt to the depression of the respiration by HCN. The effect is the same in nitrogen containing traces of oxygen. In pure nitrogen even the fusion of the pronuclei and the swelling of the sperm nucleus is prevented. The normal level of oxygen consumption is more than restored by the addition of pyocyanine to the egg suspension with HCN, *yet the division is stopped at the same stage as without pyocyanine.* The increased respiration is quite ineffective, when caused by the presence of pyocyanine. The eggs were also kept in pure nitrogen and it was found that the division is completely inhibited even in presence of pyocyanine, the concentration of which was varied from 0.006–0.02 per cent. The eggs were brought almost immediately after fertilization into Erlenmeyer flasks closed by rubber stoppers which were pierced by two glass tubes. Nitrogen, purified over heated reduced copper, was bubbled through the flasks. After two hours the bubbling of nitrogen was stopped and the stoppers removed. It was found that gradually the development started again. The rate of recovery is the same with and without the pyocyanine. Our results agree with those of Örström (cf., Runnström, 1933) according to which methylene blue does not promote the development of the *Paracentrotus* egg under anaerobic conditions. It seems that the opposite results reported by Rapkine (1929) are due to some technical error. The cell division can be completed only if the eggs are subject to anaerobic conditions after the dissolution of the nuclear membrane. Even then the block sets in again as soon as the segmentation furrow is formed. The respiration is necessary for the division of the sea urchin egg. This must mean that the respiration is coupled to processes in the cell necessary for the division. It is most likely that the reversible oxidation reduction systems acting as " carriers " (Keilin) bring about the coupling between the respiration and other processes in the cell. In

TABLE IV

Unfertilized eggs remained frozen during four days. After thawing the suspension was diluted 70 per cent with distilled water.[2]

Hexose monophosphate m.	—	0.012
Pyocyanine, %	0.006	0.006
cu. mm. O_2 in 130 min.	55	140

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[2] The undiluted suspension shows a somewhat lower oxygen uptake, probably a salt effect.

the system hemolyzed blood cells plus hexose phosphate referred to above, the pyocyanine can act as a "gear" between the respiration and the synthesis of phosphate compounds. From the reported results it must be inferred, however, that in the more complicated system, the fertilized egg cell, pyocyanine cannot act as a gear between the respiration and the processes connected with the completion of the cell division.

It is quite evident from the above data that pyocyanine is auto-oxidized in the sea urchin egg. In this respect it differs from dimethylparaphenylene diamine but resembles methylene blue according to the experiments carried out by Barron. Friedheim (1931) reports, however, that the respiration of *Bacillus pyocyaneus* enhanced by addition of pyocyanine is decreased by CO as well as by HCN. This indicates that the pyocyanine in this case does not react or reacts slowly with the molecular oxygen. It must be oxidized by the iron-containing enzyme. This difference, as compared with the conditions in the sea urchin egg may perhaps be due to differences of pH in the interior of the cells in question.

In the unfertilized egg no part of the respiration is inhibited by HCN in presence of pyocyanine. On the contrary, the respiration is considerably enhanced by the presence of HCN. This fact cannot be explained at present but it may be remembered that HCN possibly can activate certain enzymes, the action of which increase the amount of substrate present.

In the fertilized egg the part of the respiration due to the addition of pyocyanine is not inhibited by HCN. In the fertilized egg with pyocyanine there are two auto-oxidizable systems, the iron-containing enzyme and pyocyanine. This may account for the higher absolute level of respiration observed in the fertilized eggs as compared with the unfertilized ones. In the non-aged unfertilized eggs the respiration is low. The action of the iron-containing enzyme is, according to the writer's assumption, checked by a block in the chain of "carriers." Possibly a minor part of the molecules of the iron-containing enzyme is reduced (Runnström, 1930). The possibility may also be considered that some more slowly auto-oxidizable system (like Warburg's "yellow enzyme" substrate-dehydrase) may account partly or wholly for the comparatively slow oxidations in the unfertilized eggs. Anyhow, on the addition of pyocyanine to the unfertilized eggs, the chain of oxidations apparently goes wholly over pyocyanine and not or to quite an insignificant degree over the iron-containing enzyme.

The results reported above clearly show that the dehydrase-substrate system cannot be a limiting factor for the rate of respiration either in the fertilized or in the unfertilized egg.[3]

The experiments with dimethylparaphenylene diamine referred to above demonstrate, on the other hand, that the iron-containing enzyme is not the limiting factor. This points again to the "carriers" as the part of the respiratory mechanism which has to be made responsible for the considerable change in oxygen consumption following the activation of the egg.

This paper forms part of the work carried out by the writer in collaboration with Dr. L. Michaelis, and I wish to express my thanks to him for his generous help and valuable suggestions.

SUMMARY

Addition of pyocyanine to a suspension of sea urchin eggs causes an increase of the respiration of the eggs. The percentage increase is higher in the unfertilized than in the fertilized eggs. The absolute values of the oxygen consumption in presence of pyocyanine are higher after than before fertilization. The respiration of the unfertilized eggs is enhanced in the presence of pyocyanine by the addition of HCN. In the fertilized eggs, on the contrary, the respiration enhanced by pyocyanine is always decreased by HCN. Pyocyanine is auto-oxidized in the sea urchin egg; the iron-containing enzyme is not involved in the oxidations induced by pyocyanine. The rate of oxidation induced by pyocyanine is somewhat higher than that of the respiration induced by methylene blue both in intact eggs and in eggs broken up by freezing and thawing. The block of division of the sea urchin egg caused by HCN or by anærobic conditions is not removed by addition of pyocyanine. In the fertilized eggs the respiration in the presence of pyocyanine and HCN can be higher than in the control; in spite of this the division stops at the same stage as when the respiration is decreased 75 per cent by

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[3] Only the conditions prevailing in the egg immediately following the fertilization are considered here. During the ensuing development there is a gradual increase of respiration as found by Warburg (1926). The "increasing fraction" of the respiration is inhibited by lithium according to Lindahl (1933, 1934). The action of lithium on the increasing fraction of respiration can be accounted for by the law of mass action. Lithium possibly inhibits the formation of certain break-down products of carbohydrates by reacting with an enzyme or some part of an enzyme system. If this is true, the substrate-dehydrase system must gradually acquire the character of a limiting factor for the oxidation rate during the course of development. It may also be mentioned here that no increase of respiration was observed in living cells of baker's yeast on addition of pyocyanine or methylene blue. This holds true even if the respiration is inhibited by HCN. (Experiments carried out by M. Schüldt.) The situation in baker's yeast is very different from that in the sea urchin egg.

the addition of HCN alone. The general conclusion is drawn that neither the iron-containing enzyme nor the dehydrase-substrate system act as limiting factors for the oxidation rate in the unfertilized or newly fertilized egg.

LITERATURE

Barron, E. S. G., 1929. *Jour. Biol. Chem.*, **81**: 445.

Borei, H., 1934. *Zeitschr. vergl. Physiol.*, **20**: 380.

Friedheim, E. A. H., 1931. *Jour. Exper. Med.*, **54**: 207.

Friedheim, E. A. H., 1934. *Biochem. Jour.* **28**: 173.

Friedheim, E., and L. Michaelis, 1931. *Jour. Biol. Chem.*, **91**: 355.

Keilin, D., 1929. *Proc. Roy. Soc.*, Ser. B., **104**: 206.

Lindahl, P. E., 1933. *Arch. entw. mech.*, **128**: 661.

Lindahl, P. E., 1934. *Naturwiss.*, **22**: 105.

Michaelis, L., E. S. Hill, and M. P. Schubert, 1932. *Biochem. Zeitschr.*, **255**: 66.

Örström, Å., 1932. *Protoplasma*, **15**: 566.

Rapkine, L., 1929. *Compt. rend Acad. Sci.*, **188**: 650.

Runnström, J., 1930. *Protoplasma*, **10**: 106.

Runnström, J., 1932. *Protoplasma*, **15**: 532.

Runnström, J., 1933. *Protoplasma*, **20**: 1.

Runnström, J., and L. Michaelis, 1934. *Science*, **80**: 167. (Full report in press.)

Teissier, G., 1929. *Arch. de zool. expér. et gén.*, **69**: 137.

Warburg, O., 1926. Stoffwechsel der Tumoren. Berlin.

Warburg, O., 1932. *Zeitschr. angewandte Chemie*, **45**: 1. (Review.)

Warburg, O., and W. Christian, 1932. *Biochem. Zeitschr.*, **254**: 438.

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THE HEMOPOIETIC RESPONSE IN THE CATFISH, AMEIURUS NEBULOSUS, TO CHRONIC LEAD POISONING

ALDEN B. DAWSON

(*From the Biological Laboratories, Harvard University*)

In studies of the action of soluble salts of lead on fresh water fishes, Carpenter (1927, 1930) found that concentrations of lead nitrate as low as Pb 1: 3,000,000 proved lethal. The speed of lethal reaction was dependent upon the total quantity of metallic ions present as well as upon the actual concentrations used, and varied in inverse ratio to the size and weight of the fish employed. The most marked symptom was the formation of a film over the gills and skin of the fish by the interaction of the metallic ions with the surface mucus, causing death by suffocation. When insufficient lead was present the film was shed and complete recovery took place. Chemical analyses showed that no metallic ions had penetrated the body itself and Carpenter held that the action was purely an external process, chemical in type, but mechanical in its effect. In these studies the exposure to lead was of relatively short duration and probably little of the metal had entered the body, although the test (H_2S) used to detect the presence of lead in the ash residue of the body contents (Aub, Fairhall, Minot and Reznikoff, 1926) is not extremely delicate.

It seemed of interest accordingly to determine the relative efficiency of the mucus secreted by the exposed surfaces of fishes in binding the lead as an inert compound. In the light of other observations made on the effects of lead-poisoning in *Necturus* (Dawson, 1933*b*) it was felt that a biological test, the injurious effect on the circulating erythrocytes, might prove more satisfactory than the chemical detection of the metal in the tissues and body fluids of the animal.

MATERIAL AND METHODS

Young catfish 4 to 5 inches in length were used in this study. The animals were kept continuously in a bath composed of 20 cc. of 1 per cent lead acetate and 4 liters of tap water, which was changed every 48 hours. The experiments were begun in January and the experimental periods extended from 16 to 183 days. Samples of blood were drawn at regular intervals from a vein on the inner side of the operculum. The blood was studied in fresh preparation both by the Janus green-neutral red and the brilliant cresyl blue supravital techniques, and in dry smears stained by Wright's method. At the termination of the varying exposures to lead the animals and suitable controls were killed. The heart, liver, spleen, and mesonephros were removed, fixed in Helly-Zenker and stained with either hematoxylin and eosin or azur-eosin.

EFFECTS OF LEAD POISONING

Changes in the Peripheral Blood

In the catfish, practically all of the erythrocytes in the peripheral circulation are fully differentiated and basophilic or polychromatic cells are rare. In this respect they resemble those marine teleosts which are capable of removing dissolved oxygen from the water at a low oxygen tension (Dawson, 1933*a*). Exposure to lead produces no immediate or striking reaction within the blood stream. Intravascular phagocytosis of injured cells, such as occurred in *Necturus* (Dawson, 1930*a*), is almost entirely absent; the maximum number of active phagocytes ever observed in a study of any one supravital film (made with a circular cover-slip $\frac{3}{4}$ inch in diameter) being from 6 to 8. Injured cells appear smaller and become highly refractile; they are deeper in color, usually orange-yellow. Furthermore they are readily distorted, less elastic and consequently more fragile. In supravital preparations when slightly overstained with brilliant cresyl blue they appear green. With neutral red they are colored a deep red. Intracellular crystallization of hemoglobin, as in *Necturus* (Dawson, 1930*b*), is also readily induced by the slow drying of thick smears. Normally this does not occur although liberated hemoglobin crystallizes readily in this animal.

Following these early evidences of direct injury to the mature erythrocytes there is an increase in the number of polychromatic and basophilic cells, representing an exodus of incompletely differentiated erythrocytes from the erythropoietic loci, spleen, and mesonephros. At the end of thirty days a secondary anemia can be recognized and this increases in severity with the prolongation of the exposure to lead. Concomitant with the increased anemia, erythroblasts in varying stages of differentiation appear in the circulation and there is a marked increase in the number of small and large lymphocytes.

The cellular changes in the constitution of the blood, however, are not confined entirely to the cells of the erythrocytic series. Monocytes and the large granular eosinophiles are increased in number, but the most conspicuous change occurs in the numbers and relative proportions of thrombocytes and spindle cells (Fig. 9). These elements seem to be identical with the two types of cell distinguished by Jordan and Speidel (1930) in the blood of cyclostomes. The thrombocytes have a specific, fine reddish granulation with a homogeneous outer cytoplasm. The spindle cells are long and fusiform, frequently with their cytoplasmic processes recurved. They may possess granules similar to those of the typical thrombocytes. Both cells usually possess a similarly grooved nucleus. Jordan and Speidel, after reviewing the evidence on the identity of these elements, were unable to decide whether they were genetically related or independent. The typical spindle form in the catfish is not assumed in fresh preparations and the two types of cell are not readily distinguished previous to drying on the slide. It seems possible that the spindle-form is an atypical thrombocyte.

In normal blood the number of spindle cells is very small but after several weeks exposure to lead the number is greatly increased and practically equals that of the thrombocytes. This condition, when established, persisted throughout the experiments. Young thrombocytes are frequently present in considerable numbers. Some are relatively large and many give evidence of amitotic proliferation. The appearance of these atypical cells, probably all of the thrombocytic series, may be correlated with the activity of the endothelium of the heart, which will be described later. The evidence is not conclusive.

The occurrence of injured erythrocytes, the progressive anemia and the marked regenerative response in the peripheral blood indicated that large numbers of erythrocytes were being removed from the vascular channels although only a limited amount of intravascular phagocytosis could be detected. However, additional evidence of the degree of erythrocyte destruction was obtained from an examination of the liver, spleen, and mesonephros. In these organs there was a progressive storage of the hemoglobiniferous residue of degenerated red cells. In the heart no such accumulations were observed, but a marked proliferative response of the endothelium was obtained.

Changes in the Organs

In a recent study Mackmull and Michels (1932) reported on the sites and mode of storage of colloidal carbon injected into the peritoneal cavity of a teleost, the cunner. One hour after the injection, the vascular channels transported free carbon and carbon macrophages to such organs as the heart, gill, spleen, intestine, testes, ovary, and kidney. Extravascular migration of macrophages was most pronounced in the liver, spleen, kidney, and gonads.

The process of disposal of degenerate erythrocytes in the catfish parallels closely that of carbon storage in the cunner, especially in the liver, spleen, and mesonephros. Furthermore in the cunner, these organs normally contained collections of macrophages, the endogenous brown pigment of which was derived from degenerate red blood cells, and the migrating carbon macrophages displayed a selective orientation toward these areas of normal pigment deposit. However, in catfish, of the size used in this study, there are no such accumulations of stored pigment in these organs although a few isolated pigmented macrophages are occasionally found in their interstitial tissues. Accordingly the almost complete absence from the liver, spleen, and mesonephros of this pigment derived from dead erythrocytes furnishes a convenient baseline from which the amount of erythrocyte destruction may be determined by observing the progressive accumulation of pigment in the interstitial tissues by the macrophages.

Liver.—The liver of the catfish resembles that of the cunner (Mackmull and Michels, 1932), flounder, tautog, scup, minnow, and buffalo-fish (Jordan and Speidel, 1924) in that many of the veins are surrounded by sheaths of pancreatic tissue (Figs. 1 and 2). The pancreatic tissue is usually separated from the liver cells by a capillary space lined with reticulo-endothelium and frequently small foci of hemopoiesis may occur in the loose connective tissue in and about the intrahepatic pancreas. The progressive accumulation of pigment of erythrocytic origin within the liver is very marked (Figs. 3 and 4).

EXPLANATION OF PLATES

All figures are photomicrographs. Figures 1 and 3 were photographed under low power; Figs. 2, 4, 6, 7, and 8 under medium power; Figs. 5, 9, 10, 11, and 12 under oil immersion.

Plate I

1. A low power view of an area of the liver from a normal fish, showing the general appearance of the hepatic cells as well as of the intrahepatic pancreatic tissue which borders the veins of the liver.

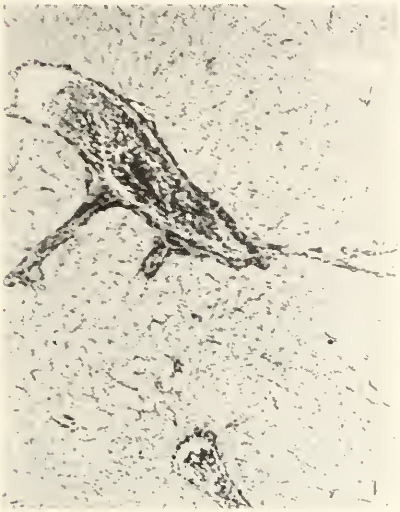
2. A portion of the above under greater magnification, showing the hepatic and pancreatic tissue in greater detail. Note the almost complete absence of brown and yellow granules (derived from disintegrated erythrocytes) in either the hepatic or interstitial cells.

3. A low power view of an area of the liver from a fish exposed to lead acetate for 183 days. The liver cells are small and dense and crowded with refractile yellow and brown granules. Masses of cells (macrophages) filled with similar pigment are scattered throughout the intertubular tissue. These cells are especially concentrated in and about the pancreatic epithelium.

4. A portion of the above under greater magnification, showing the concentration of pigment in the region of the pancreatic tissue.

The chief loci of storage are around the pancreatic cells although relatively large isolated groups of macrophages are also scattered throughout the organ between the hepatic cords. In addition to these sites of pigment storage, every liver cell is crowded with numerous brown refractive granules which appear similar to the granular contents of the macrophages (Fig. 5), but there is an almost complete absence of

Plate I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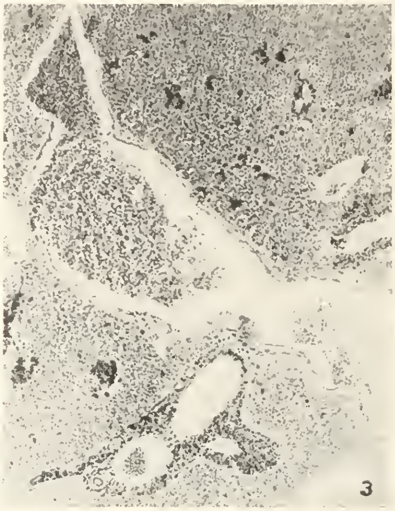
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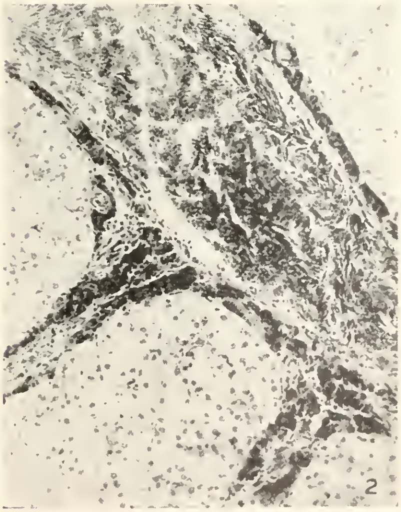
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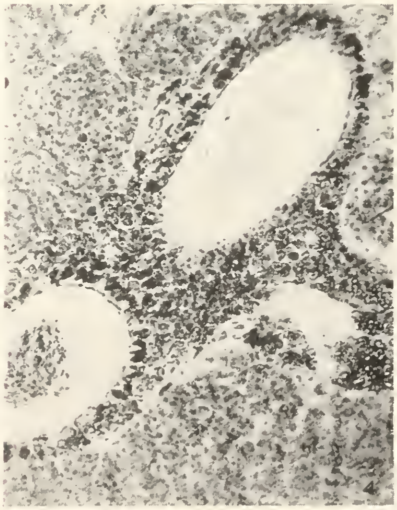
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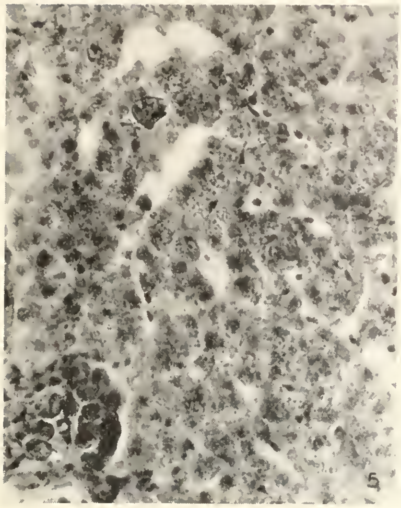
granular debris in the stellate cells of Kupffer, and in the endothelium of the veins associated with the pancreatic tissue. Only the lining of the capillaries in and about the pancreatic cells shows storage. Similar restriction of phagocytic activity of the vascular channels of the liver of the cunner was noted by Mackmull and Michels (1932) in carbon absorption. In fishes killed in late stages of lead poisoning the gall bladder is greatly dilated.

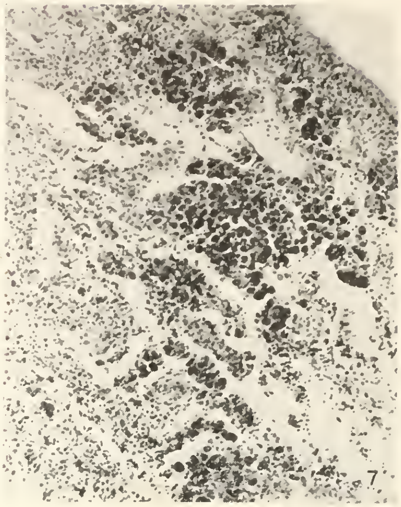
Spleen.—The general histological features of the spleen of fishes has been described by Yoffey (1929) and Mackmull and Michels (1932). The latter authors found that in the cunner the carbon macrophages and pigment macrophages migrated toward the splenic arterioles and occupied almost exclusively a periarterial position. As already pointed out in the normal young catfish, pigment macrophages are very rare, only a few isolated cells being present (Fig. 6). During lead poisoning the number progressively increases until the entire spleen is mottled with conspicuous yellow-brown masses (Fig. 7). Their distribution does not exactly coincide with that described for the cunner. Besides the definite concentration about the arterioles, there are equally large masses of pigment adjacent to the large splenic venules with which the arterioles are frequently closely associated. During lead poisoning the erythropoietic activity of the spleen is greatly reduced and few differentiating erythrocytes can be recognized in the pulp. The organ becomes smaller and paler.

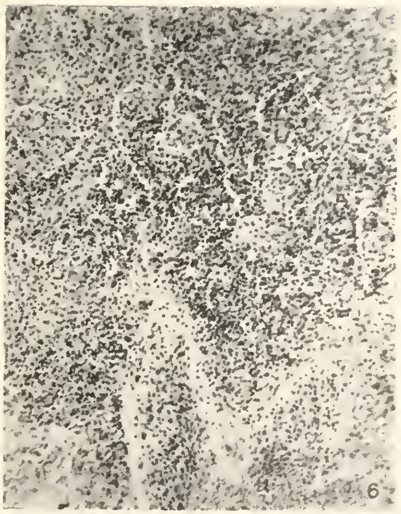
Mesonephros.—The mesonephros in the teleosts is an important hemopoietic locus in which both erythrocytes and granulocytes are produced. The greater amount of hemopoietic activity is extravascular, occurring in the uniformly distributed loose intertubular connective tissue, but many of the peritubular capillaries show sinusoidal enlargements in which differentiating blood cells are also found. Pigment macrophages show no special groupings but local accumulations of such cells are scattered rather uniformly throughout the intertubular hemopoietic tissue. The progressive storage of this pigment during the exposure to lead is as evident in the mesonephros as in the liver and spleen but is never so extensive. No pigment was ever observed in the epithelium of the uriniferous tubules.

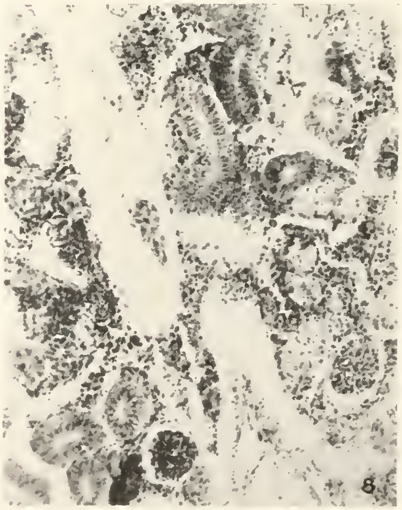
Heart.—Although pigment macrophages are commonly observed in the blood vessels of the spleen, liver, and mesonephros, they are not encountered either in the peripheral circulation or in the heart. They apparently move rapidly into the extravascular tissues of the organs and are not swept out into the general circulation. This is due largely to the fact that the degenerated erythrocytes are mostly phagocytosed by the attached reticulo-endothelial cells which later desquamate and enter the interstitial tissue.

Plate II









5. A small area of hepatic tissue, showing the compact liver cells crowded with refractile granules. An interstitial mass of macrophages is seen in the lower, left corner.

6. An area from a normal spleen, showing the absence of pigmented macrophages.

7. An area from the spleen of an animal treated with lead acetate for 183 days. Note the massive infiltration of the splenic tissue by pigmented macrophages.

8. An area from the mesonephros from the same animal, showing the intertubular, extravascular concentration of pigmented macrophages. The mesonephric tubules are free of pigment.

In many of the lower vertebrates the endothelium and even the intermuscular connective tissue may display hemopoietic potencies. In the catfish the definitive lumen of the ventricle is small and the myocardium is made up of small muscle bundles separated by vascular channels, of the order and size of capillaries, which anastomose with one another to open eventually into the main ventricular cavity. The endothelium of these ramifying vascular channels is in intimate contact with the muscle fibers and in many instances does not appear to be sharply delimited from the intermuscular connective tissue.

In the cunner (Mackmull and Michels, 1932) both the endothelium of the minute intertrabecular vessels and the intermuscular connective tissue cells phagocytosed carbon. However, phagocytosis of injured erythrocytes was never observed in the heart of the catfish at any stage of lead poisoning and, as already noted, pigmented macrophages are also typically absent from the blood channels of this organ.

In the normal catfish the hemopoietic activity of the heart appears much less than in the cunner although small numbers of differentiating cells may occur in the capillary-like spaces of the myocardium. No lymphoid sheaths were observed. After several weeks of lead poisoning a marked proliferative response was obtained and relatively large clusters of cells were found on the surfaces of the muscle bundles (Fig. 10). The cells of such clusters are elongated and radiate from the site of proliferation into the lumina of the vascular channels. Their identity has not been definitely established, but it seems likely that they give rise to the atypical elongated and spindle cells described with the thrombocytes of the blood stream (Fig. 9). Presumably these clusters of cells arise by proliferation of the endothelium and there are some indications that they are secondarily invaded by cells of the intermuscular connective tissue.

PLATE III

9. Two areas from a blood smear of a fish exposed to lead acetate for 72 days, showing atypical elongated and spindle cells.

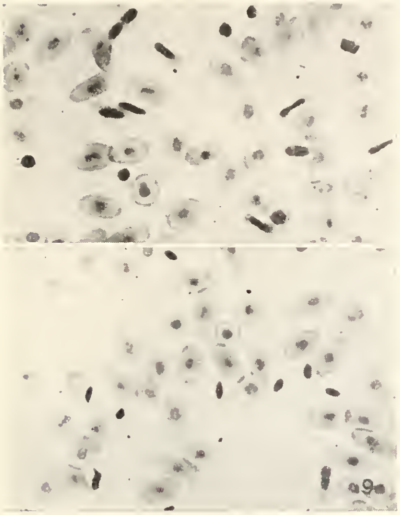
10. A region of proliferation from the surface of a muscular trabecula of the ventricle. The arrangement of cells resembles that in the margin of outgrowth of an explanted tissue. The elongated cells may give rise to the atypical cells provisionally classed with the thrombocytic series. The proliferating mass is apparently invaded by cells from connective tissue of the cardiac trabecula to form a reticular meshwork.

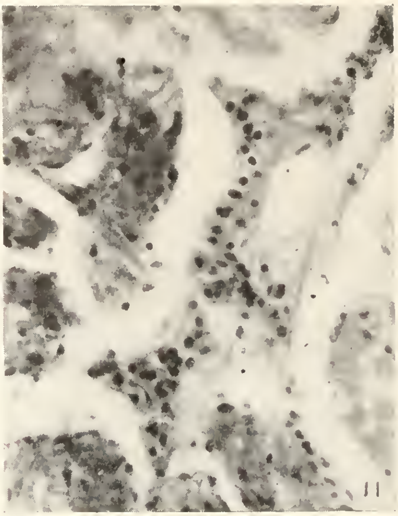
11. Another phase of the process of endothelial proliferation. The reticular meshwork is more obvious in this preparation. Small round cells and various stages of erythrocytic differentiation are present. Shrinkage has caused the separation of the tissue from the surface of the myocardial trabeculae.

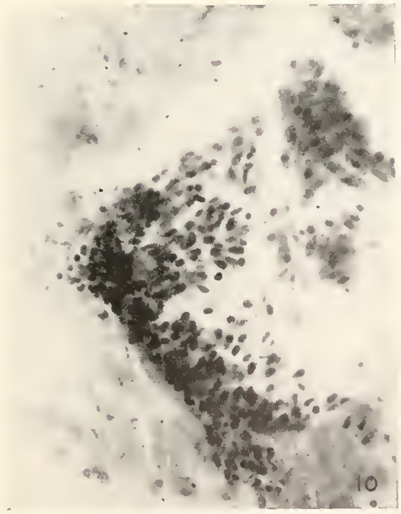
12. This has been interpreted as a late stage of hemopoietic activity. The reticular meshes are distinct and almost empty but a few small round cells and erythrocytes are present. A row of small round cells remains attached to the surface of the trabecula.

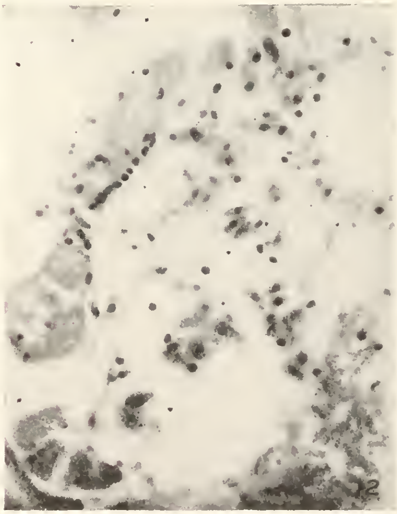
In other regions of the heart less conspicuous areas of proliferation are also present. They apparently have the same relationship with the endothelium and connective tissues as the other proliferating cells, but in the latter case various stages of erythrocyte differentiation can be readily recognized (Figs. 11 and 12). The reticular-like cells are also more readily observed in these regions. The connective tissue and

Plate III









the endothelium of the ventricular wall of the catfish appear relatively undifferentiated, being essentially mesenchymal in nature and capable of hemopoietic activity although in this study they display no phagocytic activity toward the degenerate erythrocytes.

Discussion

These experiments with lead acetate furnish abundant evidence that teleosts such as the catfish slowly absorb lead when kept in solutions of this metal for relatively long periods. The surface mucus, while it may be effective in binding the lead for short periods (Carpenter, 1927, 1930), does not adequately protect the fish during long exposures. During exposures lasting for days and even weeks, it seems probable that lead in solution would eventually be also brought into contact with the epithelium of the digestive tract as well as with that of the gills and integument, and absorption may have been favored by this circumstance. In order to reduce this possibility to a minimum the animals were always removed to fresh tap water for feeding. However, there is no reason to believe that the mucin of the digestive tract would be less efficacious in binding the metallic ions than that of the gills and epidermis, but elimination of the mucous film could not be so readily accomplished in the digestive tract.

The observations on the peripheral blood demonstrate direct injury to the erythrocytes, followed by a mild regenerative response with the eventual development of a pronounced secondary anemia. The progressive storage of pigment, derived from phagocytosed erythrocytes, by the liver, spleen, and mesonephros also furnishes corroborative evidence of a widespread destruction of red blood cells. Since direct injury to the erythrocytes is generally the first and most important sign of lead poisoning in vertebrates, it seems safe to assume that the changes described in these experiments are the direct result of the absorption of lead.

The catfish apparently is less sensitive to lead than the fishes studied by Carpenter. Lead acetate rather than lead nitrate was used exclusively in my experiments, but no lethal effects were obtained although the concentration of lead was higher than that which proved lethal in Carpenter's series.

The changes in the peripheral blood of the catfish were comparable in many respects to those observed in an earlier study of lead poisoning in *Necturus*. The chief differences were in the absence of any considerable degree of phagocytic activity in the peripheral circulation and in the appearance in the circulation of large numbers of atypical cells, elongated and spindle forms, which may belong to the thrombocytic series.

The mode of accumulation and storage by the liver, spleen, and mesonephros of the pigment derived from the injured red blood cells follows closely that described in the elimination of particulate matter from the circulation of fishes. No storage occurred in the heart but a proliferative response to the destruction of the circulating elements was obtained. The erythropoietic potentialities of the cardiac endothelium of the lower vertebrates is well known and the literature is well summarized by Mackmull and Michels (1932). Indeed, in such forms as the herring (John, 1932) it may be the initial site of erythropoiesis. There are no red blood cells in the larval stages and they appear several months after hatching.

No evidence of direct injury to the thrombocytes was obtained and it is difficult to explain either the increase in number of the cells of this series or the atypical response of the cardiac endothelium in their production. The production of thrombocytes and erythrocytes usually takes place in the same organs or tissues and the two processes go on side by side. Furthermore the two types of cell are quite similar in their plan of organization. It appears possible that any factor which depresses or modifies erythropoiesis may tend to exert a similar influence on thrombopoiesis.

SUMMARY

In the catfish, with prolonged exposure to a solution of lead acetate, definite evidence of absorption of lead was obtained. The surface mucus did not constitute an efficient barrier to the entrance of the metal as Carpenter (1927, 1930) found in more acute experiments with fishes in which a lethal reaction was frequently obtained.

The degree of destruction of the erythrocytes was used as a measure of the rate of the absorption of lead. Following the early evidences of injury to the blood cells, a mild regenerative response occurred, but eventually a pronounced secondary anemia was produced. Little phagocytosis of dead cells occurred in the peripheral circulation, but a progressive storage of the pigment derived from dead red blood cells was found in the interstitial tissues of the liver, spleen, and mesonephros. The hepatic cells also became crowded with pigment granules. No storage was observed in the heart. The pigment was accumulated chiefly in macrophages of local origin which, after ingesting erythrocytes, desquamated and migrated into the connective tissues.

Monocytes and eosinophiles were increased slightly in number in the blood stream, but the most striking change occurred in the numbers of atypical thrombocytes, and spindle cells which may belong to the thrombocytic series.

The endothelium of the heart showed marked proliferative activity. In some regions there was localized differentiation of erythrocytes. In other regions the atypical elongated cells, just referred to, were formed in large radiating clusters on the surface of the ventricular trabeculae. No explanation of the latter response to lead poisoning can be given.

LITERATURE CITED

Aub, J. C., L. T. Fairhall, A. S. Minot, and P. Reznikoff, 1926. Lead Poisoning. Medicine Monographs, Vol. 7.

Carpenter, K. E., 1927. The Lethal Action of Soluble Metallic Salts on Fishes. *Brit. Jour. Exper. Biol.*, **4**: 378.

Carpenter, K. E., 1930. Further Researches on the Action of Metallic Salts on Fishes. *Jour. Exper. Zool.*, **56**: 407.

Dawson, A. B., 1930 *a*. Intravascular Phagocytosis of Erythrocytes in Necturus Following Prolonged Immersion in Lead Acetate. *Anat. Rec.*, **45**: 345.

Dawson, A. B., 1930 *b*. Changes in the Erythrocytes of Necturus Associated with the Intracellular Crystallization of Hemoglobin. *Anat. Rec.*, **46**: 161.

Dawson, A. B., 1933 *a*. The Relative Numbers of Immature Erythrocytes in the Circulating Blood of several Species of Marine Fishes. *Biol. Bull.*, **64**: 33.

Dawson, A. B., 1933 *b*. An Experimental Study of Hemopoiesis in Necturus; Effects of Lead Poisoning on Normal and Splenectomized Animals. *Jour. Morph.*, **55**: 349.

John, C. C., 1932. The Origin of Erythrocytes in the Herring (Clupea harengus). *Proc. Roy. Soc.*, Ser. B, **110**: 112.

Jordan, H. E., and C. C. Speidel, 1924. Studies on Lymphocytes. II. The origin, function, and fate of the lymphocytes in fishes. *Jour. Morph.*, **38**: 529.

Jordan, H. E., and C. C. Speidel, 1930. Blood Formation in Cyclostomes. *Am. Jour. Anat.*, **46**: 355.

Mackmull, G., and N. A. Michels, 1932. Absorption of Colloidal Carbon from the Peritoneal Cavity in the Teleost, Tautogolabrus adspersus. *Am. Jour. Anat.*, **51**: 3.

Yoffey, J. M., 1929. A Contribution to the Study of the Comparative Histology and Physiology of the Spleen, with Reference Chiefly to its Cellular Constituents. I. In fishes. *Jour. Anat.*, **63**: 314.

THE RELATION BETWEEN CELL INTEGRITY AND BACTERIAL LUMINESCENCE

IRVIN M. KORR

(*From the Physiological Laboratory, Princeton University, and the Marine Biological Laboratory, Woods Hole, Mass.*)

In 1902 Macfadyen found that luminous bacteria exposed to liquid air would glow again on rewarming, but gave no light on rewarming if thoroughly ground at the temperature of liquid air. Harvey (1915) tried to obtain luminescence from cytolyzed and dried crushed bacteria, but even though the possibility of oxidation of the bacterial luciferin by molecular oxygen was precluded (a precaution Macfadyen did not take) by grinding the bacteria in the desiccated form or by cytolyzing them with toluol, ether, water, and other agents in the absence of oxygen, no light was obtained on moistening the ground bacteria, or aerating the cytolysate. Intact, dead, desiccated bacteria luminesced for a short time when moistened, even after prolonged extraction with fat solvents such as ether, absolute alcohol, toluol, benzine, and chloroform. These agents acting on moist or suspended bacteria, however, invariably and quickly destroyed permanently the ability to luminesce.

The demonstration of a given reaction in cellular extracts has always been a much more difficult problem in the case of bacteria than in other cells, due to the relatively greater importance of the cell structure. According to Quastel (1926) catalytic dehydrogenations, particularly, are very closely associated with the surface of the bacterial cell.

The demonstration of luminescence apart from cell structure is an even more difficult problem since it is necessary to extract both the catalyst, luciferase, and its specific substrate, luciferin. The normally close association between the two is very likely disturbed, due, conceivably, to differences in solubility between the two, to the instability of one or both apart from the normal structure of the cell, or to the oxidation of the luciferin by oxidants other than oxygen.

New methods have been applied in a continued investigation of this problem, namely, the attempt to demonstrate luminous substances from luminous bacteria destroyed in various ways. Three species were used: a fresh-water form, *Vibrio phosphorescens*, and two marine forms from Woods Hole, one unidentified, and the other, *Achromobacter fischeri*, a very brilliant variety. Four general methods, with numerous modifications, were employed for the destruction of the bacteria: (*a*) cytolysis by fat solvents, (*b*) cytolysis with hypotonic solutions (not applicable, of course, to the fresh-water form), (*c*) mechanical grinding, and (*d*) sonic radiation.

Throughout these experiments care was taken to prevent oxidation of the luciferin, and, hence, they were carried out anaerobically. After destruction of the bacteria, the suspensions were aerated in the dark and observations on the presence or absence of luminescence made with completely dark-adapted eyes.

Experiments with Fat Solvents

For this series of experiments and those involving osmotic cytolysis a double trap vessel illustrated in Fig. 1 was found convenient for the anoxic mixing of cytolytic agents with the suspension. The agent was put in one arm of the vessel (*B*), the suspension in the other (*A*), and thoroughly deaerated with a stream of hydrogen purified by passage over red-hot platinized asbestos. The two were then mixed by tilting the vessel. On aerating, no return of luminescence was observed with either chloroform or caprylic alcohol as the cytolytic agent.

A modification of this technique, in which deaeration was accomplished with sodium hydrosulfite, yielded the same results.

These confirm Harvey's (1915) results: suspensions deaerated by evacuating and then cytolyzed with toluol, ether, chloroform or carbon tetrachloride showed no luminescence on re-aerating.

Experiments with the Osmolytic Technique

The cytolytic agent in this series is diluted sea water. A volume of distilled or tap water found, in air, to cause rapid darkening of a given volume of a dense sea-water bacterial suspension is pipetted into one arm of the vessel (*A*), and the suspension into the other (*B*). The rest of the experiment is run as in the above series. Although repeated many times, luminescence has never been obtained from bacteria "cytolyzed" by hypotonic solutions in the absence of oxygen.

Darkening of bacterial suspensions by hypotonic solutions is perhaps not a true indication of complete cytolysis of the bacteria. Although the suspensions do become clear and foamy, due to the release of cellular contents almost immediately after mixing with distilled water, the bacteria remain viable for hours after complete darkening, as shown by inoculating culture media with portions of the suspension at intervals after complete darkening.[1] Excellent growth will occur after 3½ or more hours exposure to distilled water. Even though true cytolysis by this method is a very slow process, the copious release of cellular contents justifies its application.

It is possible that, despite the most rigorous exclusion of oxygen, the bacterial luciferin, during release from the cell, becomes oxidized (by the more positive systems in the cell) without giving light. Harvey (1927) has shown that *Cypridina* luciferin can undergo non-luminescent (and reversible) oxidations not involving molecular oxygen. Strong reducing agents should prevent such oxidations, but the presence of finely divided Pt in the cytolyzing mixture, which, together with the hydrogen used for deaerating, gives a reducing intensity approaching that at a hydrogen electrode, does not alter the results. The same is true if the osmotic cytolysis is allowed to take place in the presence of a

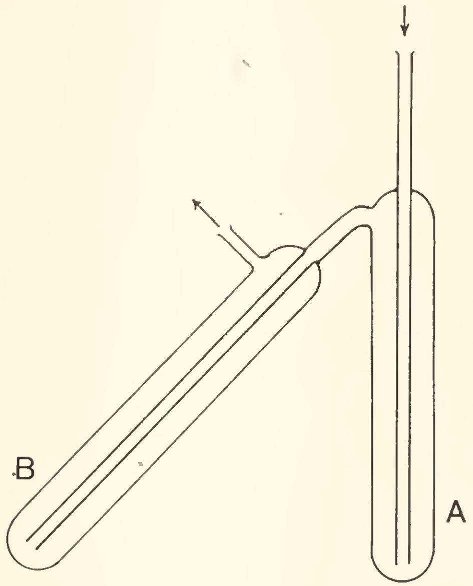


Fig. 1.

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[1] The darkening of bacterial suspensions by hypotonic solutions is doubtless at least partly an osmotic phenomenon, but not entirely, for a volume of tap or distilled water which causes rapid darkening of a given volume of suspension will do so only if added at once. If slowly poured in or added in three or four installments, even closely spaced, the time for darkening is greatly extended, the suspension retaining some luminescence for hours. Another recent and pertinent observation is that the destruction of the capacity for luminescence by hypotonic solutions is very much more rapid under anaerobic conditions than in aerobic. The difference is far too large to be ascribed merely to a difference in the rates of penetration of the water in the presence and absence of oxygen.

small but adequate mount of neutralized sodium hydrosulfite. *Cypridina* oxyluciferin, which gives no light with luciferase, can be reduced by hydrosulfite to luciferin, which does give light with luciferase.

Another possible cause of failure to obtain luminescence from substances liberated from bacteria is that the photogenic substances become diluted or separated, even when liberated into small volumes of liquid, whereas, in the cell, they are quite concentrated and closely associated. The attempt was made in the following experiments to minimize this dispersion by concentrating the substances on adsorbing surfaces. The procedure was exactly that described above for the osmolytic technique, except that an adsorbent, either $Fe(OH)_3$, kaolin, or Norit A was added to the distilled water. For the use of $Fe(OH)_3$ the bacteria had to be suspended in isotonic sucrose to prevent the too rapid precipitation of the sol by the salts of sea water on mixing. In no case was luminescence observed on aeration.

Repetition of these experiments but with the addition of finely divided Pt to both tubes to provide reducing action (together with the H_2) gave the same results.

In some experiments the adsorbents were replaced by fresh-water luminous bacteria from 4–6-day-old cultures which had ceased luminescing, or other fresh-water (non-luminous) forms. But the presence of normal bacterial surfaces instead of those of the colloids did not affect the results.

Attempts were also made to reduce oxyluciferin, liberated from cells by aerobic cytolysis. A thick mass of bacteria (*A. fischeri*), washed and collected by centrifuging, was suspended in water, in aqueous colloidal $Fe(OH)_3$ or in aqueous kaolin suspensions, in air. Some time after complete darkening of the suspension (due to hypotonicity) it was again centrifuged, the supernatant fluid poured off and the thick gelatinous mass at the bottom of the tube quickly dried in vacuo over $CaCl_2$. The dried powder was suspended in water and, in the absence of oxygen, exposed to the action of colloidal $Pt + H_2$. This (again assuming a fundamental similarity between bacterial and *Cypridina* luciferin) should have reduced the oxyluciferin formed during its (aerobic) release from the cell to luciferin. However, no light appeared on aeration in any case.

AUTOLYTIC METHOD

A dense mass of *A. fischeri* was kept at 38° C. for 3 days in order that autolysis might occur. This material, after reduction with $Pt + H_2$ or with hydrosulfite, gave no luminescence on aeration. The autolysate, which should have contained large quantities of bacterial luciferase (and oxyluciferin), when mixed with bacterial luciferin, i.e. with what would be analogous to a *Cypridina* luciferin preparation (bacteria boiled and cooled in a hydrogen atmosphere), gave no light. Attempts to demonstrate luciferase in ground bacteria, desiccated marine bacteria resuspended in water or adsorption extracts, described in the preceding paragraph, with the above luciferin preparation also proved negative. Likewise, "cross" experiments with *Cypridina* luciferin and luciferase also failed.

Extracts of *Cypridina* which have become dark due to the oxidation of all the luciferin will, when electrolyzed, luminesce in the vicinity of the cathode, due to cathodal reduction (Harvey, 1923). Various extracts of the luminous bacteria, similarly treated, did not luminesce.

Mechanical Destruction of Bacteria

This was accomplished with a device consisting of a ground-glass rod revolving in a close-fitting glass tube whose inner surface in contact with the rod is also ground.[2] (Ten Broeck, 1931; Glaser and Coria, 1935.) The rod was fitted to the shaft of a small electric motor. In order to grind in the absence of oxygen, the grinder and motor were hermetically enclosed in a casement of brass and glass. The glass part consisted of a test tube sealed with deKhotinsky cement into a circular opening in the brass case containing the motor. This glass tube contained the grinding parts, thus permitting observation. Wires to the motor and gas-inlet and -outlet tubes were fitted to the brass case through a rubber stopper, so that the apparatus could be exhausted with a vacuum pump or hydrogen passed through it while grinding was taking place.

A small quantity of thick, bright suspensions was carefully pipetted into the bottom of the grinding vessel without getting bacteria on the walls of the vessel. The bacteria were ground either in an atmosphere of hydrogen or in a vacuum at a high rate of speed for one to two hours, after which air was admitted in the dark and observations made. The temperature never rose above 28° C. If grinding was thorough, no light appeared on aerating. If, before grinding was begun, finely divided Pt was added to the suspension, and the experiment performed in a H_2 atmosphere, the results were also the same, no light appearing on aeration.

Experiments with Sonic Vibration

A number of experiments in which destruction of the bacteria was accomplished by means of a powerful magnetostriction oscillator of

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[2] The author is grateful to Dr. R. W. Glaser of Rockefeller Institute, Princeton, for a set of the glass parts, after which others were modelled.

9,000 cycles were performed.[3] Sonic radiation from the apparatus used is known to break apart bacteria into fragments (Chambers and Gaines, 1932) and will extinguish the luminescence of bacterial suspensions in air in 13 to 20 minutes. The suspension was contained in a glass vessel fitted over the vibrating nickel-alloy tube, and deaerated by a stream of nitrogen first passed over heated copper to remove traces of oxygen. The suspension was completely darkened by the removal of dissolved oxygen before the generator was turned on, but for some reason the suspension became slightly luminous again on even momentary irradiation, indicating the presence of a small amount of oxygen. Although this was washed out by the continued stream of N_2, there was almost always a reappearance of luminescence on irradiating. Bacteria, irradiated in the presence of this small concentration of oxygen for 13–20 minutes (the time required depending on the concentration of bacteria) showed no luminescence on blowing air through their suspensions. In only two experiments were we successful in completely deaerating the suspensions (as shown by darkness of the suspensions on momentarily turning on the oscillator). These, also, did not luminesce on being aerated at the end of the run.

The work of Shoup (1929, 1933, 1934) and of Taylor (1932, 1934) has shown that when respiration is inhibited by a variety of methods, e.g. KCN, CO, excessive dinitrophenol, lowered oxygen tension, luminescence is not affected until respiration is reduced to about 40–50 per cent of the normal, when the intensity drops off. Whether the failure of respiration and of luminescence is due to some common factor or whether the failure of respiration bears some causal relation to that of luminescence cannot be readily determined. It is easy to show that the respiration of "cytolyzed" bacteria is greatly affected. As determined by a modification of the dimming-time method their respiration is reduced to far below the 40–50 per cent level, often lower than 10 per cent. It seems that the dehydrogenases are the affected systems, rather than the oxidase-cytochrome system, for the rate of reduction of methylene blue (Thunberg technique) is greatly lowered by osmotic cytolysis while the Nadi test for indophenol oxidase is, if anything, intensified.

Discussion

Even a cursory review of the literature shows that bacterial luminescence is far from unique among biological oxidative reactions in being dependent upon cellular structures and having catalysts not extractable in the soluble form. Some of the oxidative reactions of blood cells are inhibited or destroyed by osmotic hemolysis, and it is particularly true that the dehydrogenations by bacteria disappear with cell destruction (Quastel and Woolridge, 1927, 1928; Stephenson, 1928; Young, 1929), although some of the dehydrogenations are less dependent on normal structure than others. Other interesting observations are those of Gozony and Suranyi (1925) and of Shwartzman (1926), who found that lysis with phage greatly reduced the rate of methylene blue reduction by bacteria. Clifton (1933) found that lysis with phage causes less negative reduction potentials in suspensions of *Staphylococcus aureus*. I have found that cytolysis (by hypotonic solutions, cytolytic agents, and autolysis) produces similar effects on luminous bacteria. The oxidative reactions observed by Neill and his coworkers (1924–1928) in detritus-free extracts of *Pneumococcus* are all very likely due to the presence of non-enzymic catalysts such as certain intracellular reversibly oxidizable pigments.

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[3] I am most grateful to Dr. Leslie A. Chambers and the Johnson Foundation for Medical Physics for the use of the oscillator and assistance in its operation.

Whether luciferase can be classed with the dehydrogenases is, of course, debatable. One fundamental similarity is shown, however, in that luciferin (LH_2) is oxidized to oxyluciferin (L) by a dehydrogenation catalyzed by luciferase. The energy derived from this oxidation is then transferred to the luciferase, which luminesces (Harvey, 1927). *Cypridina* luciferin, however, differs from other hydrogen-donators in being autoxidized in the presence of oxygen, and readily oxidized by certain oxidizing agents, e.g. ferricyanide and quinone, but without the production of light. Hence, for luminescence, the reaction is highly specific.

Although bacterial luminescence and *Cypridina* luminescence are both dependent on molecular oxygen, the inability to demonstrate the luciferin-luciferase reaction or to obtain any luminescence from injured or disintegrated bacterial cells, even under the most favorable conditions, forces us to the conclusion that luminescence in these forms is very decidedly bound up in an intimate manner with structural conditions, presumably unaltered surfaces in or on the cell.

SUMMARY

Three species of luminous bacteria were injured or destroyed by a variety of modifications of four general methods: (*a*) cytolysis with fat solvents, (*b*) osmotic cytolysis, (*c*) mechanical grinding, and (*d*) intense sonic vibration. Although all experiments were performed under conditions which would have prevented the oxidation of the bacterial luciferin and which were, in general, favorable for bioluminescence, it was not possible in any case to demonstrate the luciferin-luciferase

reaction or obtain luminescence from bacteria whose structure had been materially altered. The conclusion is drawn that bioluminescence, like many other bacterial oxidative phenomena, is closely associated with cellular structure. Respiration and reducing activity were shown also to be greatly affected.

The author wishes to express his profound gratitude to Professor E. Newton Harvey, under whom this work was done, for his interest and helpful advice.

LITERATURE CITED

Chambers, L. A. and N. Gaines, 1932. Some Effects of Intense Audible Sound on Living Organisms and Cells. *Jour. Cell. Comp. Physiol.*, **1**: 451.

Clifton, C. E., 1933. Oxidation-Reduction Potentials in Cultures of Staphylococcus aureus. *Jour. Bact.*, **25**: 495.

Glaser, R. W., and N. A. Coria, 1935. The Culture and Reactions of Purified Protozoa. *Am. Jour. Hyg.*, **21**: 111.

Gózony, L., and L. Surányi, 1925. Reduktionsversuche mit Bakteriophagen. *Centralb. f. Bakt., Parasit., u. Infekt. I Abt. Originale*, **95**: 353.

Harvey, E. N., 1915. Studies on Light Production by Luminous Bacteria. *Am. Jour. Physiol.*, **37**: 230.

Harvey, E. N., 1923. Studies on Bioluminescence. XV. Electro-reduction of Oxyluciferin. *Jour. Gen. Physiol.*, **5**: 275.

Harvey, E. N., 1927. Bioluminescence. *Bull. Nat. Res. Council*, **59**: 50.

Macfadyen, A., 1902. On the Influence of the Prolonged Action of the Temperature of Liquid Air on Micro-Organisms and on the Effect of Mechanical Trituration at the Temperature of Liquid Air on Photogenic Bacteria. *Proc. Roy. Soc.*, **71**: 76.

Neill, J. M. et al, 1924–28. Papers on Cell-Free Extracts of Pneumococcus. *Jour. Exper. Med.*, vols. **40–47**.

Quastel, J. H., 1926. Dehydrogenations Produced by Resting Bacteria. IV. A Theory of the Mechanism of Oxidations and Reductions in Vivo. *Biochem. Jour.* **20**: 166.

Quastel, J. H., and W. R. Woolridge, 1927. Experiments on Bacteria in Relation to the Mechanism of Enzyme Action. *Biochem. Jour.* **21**: 1224.

Quastel, J. H., and W. R. Woolridge, 1928. Some Properties of the Dehydrogenating Enzymes of Bacteria. *Biochem. Jour.* **22**: 689.

Shoup, C. S., 1929. The Respiration of Luminous Bacteria and the Effect of Oxygen Tension upon Oxygen Consumption. *Jour. Gen. Physiol.*, **13**: 27.

Shoup, C. S., 1933. Luminescence and Respiration of Bacteria in Carbon Monoxide. Abstr. *Biol. Bull.*, **65**: 370.

Shoup, C. S., and A. Kimler, 1934. The Sensitivity of the Respiration of Luminous Bacteria for 2, 4-Dinitrophenol. *Jour. Cell. Comp. Physiol.*, **5**: 269.

Shwartzman, G., 1926. La Réduction du Bleu de Méthylène dans L'Autolyse Transmissible. *Comp. Rend. Soc. Biol.*, **95**: 431.

Stephenson, M., 1928. On Lactic Dehydrogenase; a Cell-Free Enzyme Preparation Obtained from Bacteria. *Biochem. Jour.*, **22**: 605.

Taylor, G. W., 1932. The Effects of Hormones and Certain Other Substances on Cell (Luminous Bacteria) Respiration. *Jour. Cell. Comp. Physiol.*, **1**: 297.

Taylor, G. W., 1934. The Effect of Narcotics on Respiration and Luminescence in Bacteria with Special Reference to the Relation between the Two Processes. *Jour. Cell. Comp. Physiol.*, **4**: 329.

Ten Broeck, C., 1931. A Simple Grinder for Soft Tissues. *Science*, **74**: 98.

Young, E. G., 1929. Endocellular Enzymes of Bacillus coli communis. *Biochem. Jour.*, **23**: 831.

THE EFFECTS OF ANTUITRIN S AND SHEEP PITUITARY EXTRACT ON THE FEMALE LIZARD, ANOLIS CAROLINENSIS

LLEWELLYN THOMAS EVANS

(*From the Biological Laboratories, Harvard University*)

Introduction

The relationship between the pituitary and the female genital system of the reptile is only partially understood. Ovulation in the snake, *Xenodon merremi*, resulted after five homoplastic pituitary implants (Houssay, 1931). Ovulation was induced in *Anolis carolinensis* after five homoplastic pituitaries were implanted and in one case after three frog pituitaries were implanted (Evans, 1935). Forbes (1934) reports hypertrophy of the genital system of young alligators with whole gland sheep pituitary extract.

Since homoplastic pituitary implants induce ovulation in reptiles, the present study was made in order to learn whether mammalian pituitary extracts would also induce ovulation.

The oviduct of the normal female of *Anolis carolinensis* is divided into five general regions: the infundibulum, the tube, the albumen-secreting portion, the uterus, and the vagina (Giacomini, 1893). The epithelial cells of the albumen-secreting portion are cuboidal and seem to be the only glands present, there being none beneath the mucous layer. Special glands, however, exist in the submucous region of the uterus. These connect with the lumen of the uterus by means of ducts.

Materials and Description

Antuitrin S Series

Fifty-five females of *Anolis carolinensis* received injections of Antuitrin S (Parke Davis, Series 3024898). The first injections were administered on November 22, 1933, the last on April 1, 1934. Some females received more injections than others and at varying intervals, but the dosage in all cases was .02 cc. diluted with two or three parts of cold-blooded Ringer's solution. This dose represents the maximum that was safe to use. Larger dosage often proved fatal. Twenty-five females served as controls and were kept under the same conditions as the injected animals.

Internal changes brought about by the injections were largely confined to the ovaries and oviducts. Figure 1*a* shows the control (on the left) as compared to 1*b* from a female which received three daily injections, was then rested for 30 days, followed by six more injections, then another rest of 7 days followed by two injections. She was killed 24 hours later, February 28, 1934, together with the control shown in Fig. 1. Figure 1*c* shows the condition produced in a female which received twelve daily injections and then was killed, 24 hours later, March 15, 1934. These two particular cases are representative of the results obtained in the 55 females, so it seems unnecessary to illustrate other cases.

Reference to Fig. 1 reveals the alteration produced in the oviducts by injections. In the control the oviduct appears as a continuous straight tube which becomes slightly larger in the region of the uterus. Figures 1*b* and 1*c*, however, show the oviduct very much enlarged and thrown into many folds. The albumen-secreting portion becomes greatly lengthened but its walls remain relatively thin. The uterus, on the contrary, increases more in circumference than in length. This increase in diameter is brought about partly by a thickening of the walls due to the greatly hypertrophied glands lying beneath the mucous lining of the lumen. The infundibulum (*i*), albumen-secreting portion (*al*), and the uterus (*u*) are well shown.

The lumen of the infundibular region is usually closed by the temporary fusion of the mucous walls but, under the influence of the extract, the lumen becomes patent. The epithelial cells lining the tube region are not visibly hypertrophied by the hormone. The mucosa of the albumen-secreting portion shows considerable response. The individual cells change in shape from cuboidal to columnar.

EXPLANATION OF PLATE I

Abbreviations: *al*, albumen-secreting portion of oviduct; *g*, glandular area of uterus or shell-secreting portion of oviduct; *i*, infundibulum of oviduct; *l*, lumen; *m*, smooth muscle layer of oviduct; *s*, mucous layer of oviduct.

All figures are of *Anolis carolinensis*.

FIG. 1. 4¾ ×. Ovary and oviduct. (*a*) Control. Killed February 28, 1934. (*b*) Received injections of Antuitrin S with intervals of rest as follows: three daily injections, 30 days rest, 6 daily injections, 7 days rest, 2 daily injections. Killed February 28, 1934. (*c*) Received 12 daily injections of Antuitrin S. Killed 24 hours later, March 15, 1934. (*d*) Received 12 injections of whole gland sheep pituitary extract at 36-hour intervals. Killed 24 hours later, March 15, 1934.

FIG. 2. 210 ×. Control. Cross-section of albumen-secreting portion of the oviduct.

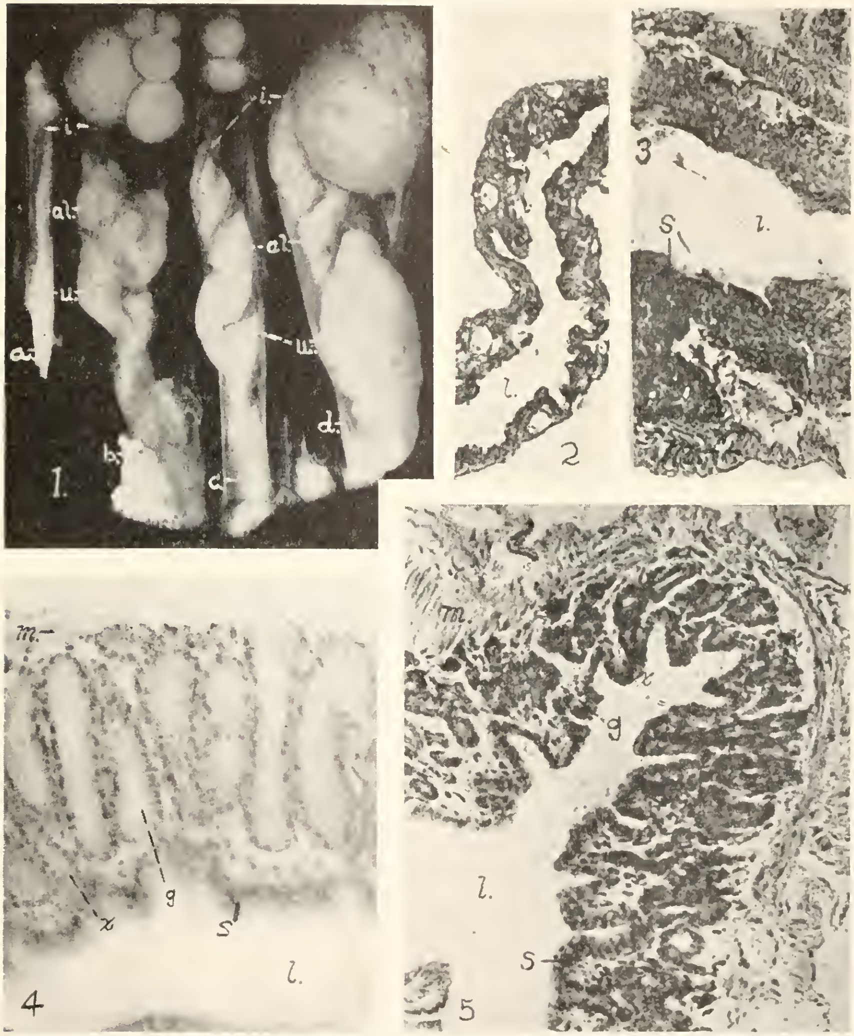
FIG. 3. 210 ×. Same as *d* in Fig. 1. Cross-section of albumen-secreting portion of the oviduct.

FIG. 4. 210 ×. Same as *d* in Fig. 1. Cross-section of uterus. Note duct from glandular area at *x*.

FIG. 5. 210 ×. Control. Cross-section of uterus. Note duct from glandular area at *x*.

The uterus shows a curious contrast to the albumen-secreting portion in its response to the injections. The superficial cells of the mucosa are not altered in shape but remain cuboidal. The so-called

Plate 1



shell-secreting glands which lie between the mucous layer and the muscle layer become much enlarged.

The ovary is definitely affected by Antuitrin S as Figs. 1*a*, 1*b*, and 1*c* bring out clearly. Figure 1*c* shows approximately 100 per cent hypertrophy as compared with the control. In Fig. 1*b*, however, the ovary is at least four times larger than the control.

It is interesting to note that eleven injections in groups of 3, 6, and 2, with several days interval between injections, produced a greater hypertrophy of both ovary and oviduct (1*b*) than was secured when one injection was given daily for twelve days (1*c*). Seasonal effects can be ruled out since 1*b* was killed two weeks earlier than 1*c*.

No matter what combination of injections and rest intervals was used, Antuitrin S failed to induce egg-laying.

Sheep Pituitary Series

Twenty-five females of *Anolis carolinensis* were injected with sheep pituitary (whole-gland pituitary extract [1]) at varying intervals between December 1, 1933 and April 1, 1934 and with a varying number of injections. A single dose was always .02 cc. diluted as for Antuitrin S. The same controls were used.

The use of sheep pituitary gave results which were more satisfactory in that complete ovulation was induced. On March 23, 1934 two eggs were laid and on March 24, two more. A fifth appeared on April 11. In these particular cases, twelve injections of sheep pituitary were given at an average of 36-hour intervals, the last injection being on March 2, 1934. All of the 25 females that received the sheep pituitary responded by a great hypertrophy of the ovaries and oviducts. Many cases showed, however, that the eggs which were ready to be laid were retained in the ovary. Such females had unusually large abdomens, moved about very little, and seemed to be in a lethargic state. In these cases the eggs were very slowly resorbed so that by the first of August the ovaries were reduced to the size of that shown in Fig. 1*c*. The bright orange color of the partially resorbed eggs made them easily distinguishable from young ova of the same size which were always white.

Figure 1*d* shows a typical case of induction. This particular animal was killed March 15, 1934, 24 hours after receiving twelve injections at 36-hour intervals. The large egg is almost ready to leave the ovary. The infundibulum has surrounded the egg, enveloping it like a transparent membrane. The latter is visible in the fresh condition only because of its blood vessels, which form a network over the egg (not visible in the photograph). The same figure shows clearly the infundibulum, albumen-secreting portion, and the uterus. Comparison with the normal oviduct at the time of ovulation (not shown) makes it seem certain that this figure represents a condition homologous to that just prior to normal ovulation.

Figure 1*d* represents, then, the maximum degree of hypertrophy which was obtained in our injected series. The uterine portion is especially large. This hypertrophy is due primarily to the increase in the size of the glands which lie in the submucous region. These are larger in all dimensions than the same glands in Fig. 1*b* or 1*c*. Figure 4 shows a section of this uterus in greater detail. The glandular cells are palisade in shape and are filled with a highly refractive granular substance. The nuclei lie at the outer periphery of the cells and away from the lumen of the gland. The cells have become so enlarged that the free secreting surfaces of opposite cells abut one another, thus closing the lumina of the glands.

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[1] Kindly supplied by Dr. Oliver Kamm of Parke Davis Company.

Non-sexual Effects of Both Antuitrin S and Sheep Whole Gland Extract

These effects made their appearance after the third or fourth injection and continued for at least four months after the last injection. Both extracts caused the females to differ from the controls in that (1) their appetites were greater and while they ate more they remained much thinner; (2) general activity and speed of movement were greater; (3) moulting occurred oftener.

Summary

Between November 1933 and April 1934, 55 females of *Anolis carolinensis* were injected with Antuitrin S, while 25 were injected with whole gland sheep pituitary extract. Twenty-five females served as controls. The dosage was .02 cc. of either extract diluted with cold-blooded Ringer's solution.

Both extracts caused hypertrophy of the ovaries and oviducts but ovulation and egg-laying were induced only with sheep pituitary. Moreover, in many females which received sheep extract the ovaries contained mature ova that were not laid but were slowly resorbed during the ensuing spring and summer.

With both extracts the epithelial cells lining the albumen-secreting portion of the oviduct changed from cuboidal to columnar, while those of the epithelium of the uterus were very little affected by the injections. The deep-lying shell glands of the uterus, however, were greatly enlarged.

Injected animals ate more, were more active, and moulted oftener than controls.

I wish to thank Professor Leigh Hoadley and Professor Alden B. Dawson for their kind help and valuable criticism during the course of this investigation.

LITERATURE CITED

Evans, L. T., 1935. The Effects of Pituitary Implants and Extracts on the Genital System of the Lizard, Anolis carolinensis. *Science*. In press.

Forbes, T. R., 1934. Effect of Injections of Pituitary Whole Gland Extract on Immature Alligator. *Proc. Soc. Exper. Biol. and Med.*, **31**: 1129.

Giacomini, E., 1893. Sull' ovidutto die Sauropsidi. *Monit. Zool. Ital.*, **4**: 202.

Houssay, B. A., 1931. Action sexuelle de l'hypophyse sur les poissons et les reptiles. *Compt. Rend. Soc. Biol.*, **106**: 377.

THE HEMOLYTIC ACTION OF PHOTOFLUORESCEIN [1]

JOHN F. MENKE

(From the Department of Embryology, Carnegie Institution of Washington, Baltimore, Maryland)

Numerous efforts have been made to offer an explanation for photodynamic action. The most recent is that of Blum (1930), who obtained hemolysis of red blood cells with a previously irradiated solution of eosin, and sought to explain it by demonstrating the production of a peroxide during the irradiation. Later Blum and Spealman (1933) were unable to substantiate this by further experiment.

During my work on the effect of photodynamic action on normal and malignant cells, I began to suspect that possibly the product of irradiation of these dyes as described by Wood (1922) might be in some way responsible for this action. With this in mind the following method was devised for the making and the isolation of the photocompound of fluorescein.

A photocompound of fluorescein was made by continuous irradiation of a dilute solution of sodium fluorescein in distilled water; 50 milligrams of the dye were dissolved in a two-liter Erlenmeyer flask of distilled water. Rays of sunlight were concentrated to a point focus on the flask by means of a 4½-inch reading glass. At first the solution fluoresces brilliantly, but slowly diminished until after some 300 hours of irradiation the fluorescence of the solution completely disappears, leaving a product which Wood calls the photocompound of fluorescein or photofluorescein. Wood described this compound as being an intermediate product in the bleaching reaction of the dye, and states that further continued irradiation will result in bleaching.

In order to reclaim the photocompound from this very dilute solution, it was precipitated by the addition of a small amount of hydrochloric acid and separated by filtering. After thorough washing with distilled water the precipitated dye was made back into its sodium salt by the careful addition of barely enough sodium hydroxide to carry it into solution. Then by careful evaporation the crystalline material was obtained, weighed, and dissolved in Locke's solution in quantity such that 0.1 cc. contained one milligram of photofluorescein. Glass electrode pH determination to check the possibility of excess alkali was

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[1] Aided by a grant from the International Cancer Research Foundation to Dr. W. H. Lewis.

made on a 1/1,000 dilution in Locke's solution. The pH was found to be 7.85.

Red blood cells were obtained from normal healthy rats. Heparin was used to prevent clotting. The plasma was removed after centrifugation and the cells washed thoroughly with Locke's solution. Various alkaline buffer solutions were used in control experiments to rule out any effect of the slight alkaline reaction of the photocompound. All experiments were carried out in a water bath in which the temperature was controlled at 38° C.

Nine series of experiments were set up to test the hemolytic action of this photocompound and to provide the necessary control tests.

Series 1. To 1.0 cc. of 1 per cent red blood cells in Locke's solution was added 1.0 cc. of Locke's solution containing 1.0 milligram of photofluorescein. This was incubated in the dark for two hours at 38° C. Complete hemolysis resulted.

Series 2. To 1.0 cc. of 1 per cent red blood cells in Locke's solution was added 1.0 cc. of Locke's solution containing 1.0 milligram of (ordinary) fluorescein. This was incubated in the dark for two hours at 38° C. No hemolysis resulted.

Series 3. Same as Series 2 except that this was incubated in the presence of strong sunlight for 2 hours at 38° C. Complete hemolysis resulted.

Series 4. One cc. of 1 per cent red blood cells in Locke's solution was centrifuged and the Locke's solution drawn off. To the remaining red blood cells was added 2 cc. of Locke's solution containing 1.0 milligram of fluorescein which had been previously irradiated with sunlight for 2 hours. The resulting combination of red blood cells and the previously irradiated dye in Locke's solution was then incubated in the dark for 2 hours at 38° C. Partial hemolysis resulted.

Series 5. To 1.0 cc. of 1 per cent red blood cells in Locke's solution was added 1.0 cc. of Locke's solution buffered to pH 8.0. This was incubated in the dark for 2 hours at 38° C. No hemolysis resulted.

Series 6. Same as Series 5 except that the 1.0 cc. of Locke's solution added was buffered to pH 9.0. This was incubated in the dark for 2 hours at 38° C. No hemolysis resulted.

Series 7. Same as Series 5 except that the 1.0 cc. of Locke's solution added was buffered to pH 10.0. This was incubated in the dark for 2 hours at 38° C. Slight hemolysis resulted.

Series 8. To 1.0 cc. of 1 per cent red blood cells in Locke's solution was added 1.0 cc. of Locke's solution. This was incubated in the dark for 2 hours at 38° C. No hemolysis resulted.

Series 9. Same as Series 8 except that this was incubated in the

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presence of strong sunlight for 2 hours at 38° C. No hemolysis resulted.

From the results given it can be seen that photofluorescein is capable of producing hemolysis in the dark to the same extent that fluorescein is capable in the light, and that the unirradiated fluorescein has no apparent hemolytic action in the dark. Also, that a short two-hour previous irradiation of a solution of the dye produces enough of the photocompound to produce a partial but well marked hemolysis. The buffer solution controls showed no hemolysis except in the case of pH 10.0, in which slight hemolysis occurred. This result rules out any possible effect of the pH 7.85 reaction of the photofluorescein. By a series of trials it was found that approximately two hours in the dark were required for a 1:2,000 dilution of the photofluorescein to hemolyze one cc. of 1 per cent red blood cells.

It is logical to suppose that although long periods of irradiation are necessary for the formation of appreciable quantities of the photocompound, shorter periods of irradiation will produce sufficient amounts to produce hemolysis when the cells are in contact with the dye during the irradiation, or when the dye is previously irradiated. Just what changes are induced in the molecule by the irradiation are as yet unknown. Wood has shown that the absorption spectrum of the photofluorescein has shifted toward the red end of the spectrum.

BIBLIOGRAPHY

BLUM, H. F., 1930. Studies of Photodynamic Action. I. Hemolysis by previously irradiated fluorescein dyes. *Biol. Bull.*, **58**: 224.

BLUM, H. F., AND C. R. SPEALMAN, 1933. Photochemistry of Fluorescein Dyes. *Jour. Phys. Chem.*, **37**: 1123.

MENKE, J. F., 1934. Photodynamic Action on Normal and Malignant Cells in Vitro. *Carnegie Inst. Wash.*, Publ. No. 457. Contributions to Embryology, Vol. 25.

WOOD, R. W., 1922. Fluorescence and Photochemistry. *Phil. Mag.*, **43**: 757.

THE VALIDITY OF THE CENTRIFUGE METHOD FOR ESTIMATING AGGREGATE CELL VOLUME IN SUSPENSIONS OF THE EGG OF THE SEA-URCHIN, ARBACIA PUNCTULATA

HERBERT SHAPIRO[1]

(From the Physiological Laboratory, Princeton University, and the Marine Biological Laboratory, Woods Hole, Massachusetts)

A problem which frequently arises in biological work is the determination of the total volume of the cells whose functional activities are being studied. This appears, for example, in measurements of oxygen consumption (Whitaker, 1933; Gerard and Rubinstein, 1934), where it may be desirable to give absolute, rather than relative figures. With free cells whose outline closely approximates that of a sphere, such as certain marine eggs, it is possible to estimate the total volume of material to a degree of precision depending upon the regularity of configuration of the particular species of cell, by measuring the diameter of the cells, and the concentration of cells with a suitable counting chamber. This procedure cannot be followed when cells are of irregular outline, for then the volume of the individual cell is not easily computed; one must then resort to a method such as centrifugalization. The problem then becomes one of ascertaining how closely the volume of the packed cells, as estimated from the length of the capillary occupied by the mass of material, approaches the true volume, and determining the complicating effect of extracellular structures such as enveloping membranes, or other layers of varying dimensions. A special case is considered here, viz., that of the egg of the Woods Hole sea-urchin, *Arbacia punctulata*. A justification for a detailed inquiry of a largely routine nature lies in the wide use of these cells for physiological work. Harvey (1932) has collocated the quantitative data pertaining to the various aspects of the chemical and physical properties of this cell. No validation of the centrifuge method has appeared, although numerous investigators have used it for this and other cells to determine egg volume. The *Arbacia* egg is good material for this study because, owing to its negligible departure from perfect sphericity of shape, its volume can be computed fairly precisely from a measurement of the egg diameter; and the material is obtainable in abundance. To consider the limits of error in the use of the centrifuge, under the conditions specified, for a measure of the aggregate volume of cell populations, the evidence will be given under these general headings: methods of measuring concentrations and volume (hemacytometer and centrifuge), distribution of egg sizes, the stacking of cells, extracellular structures, the general effects of centrifugalization on the packing of cells, and on their subsequent viability.

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[1] National Research Council Fellow in the Biological Sciences.

Relative Advantages of Counting and Centrifuging

In weighing the relative advantages of the two methods it is to be noted that the centrifuge method cannot be used where the volume of cells must be known before they are used, or where it is desired to follow the effect of experimentally imposed conditions upon embryological development after measurements have been made. The hemacytometer method, on the other hand, although slightly more laborious, and involving measurement of cell diameters and computation of their volumes, requires only small samples of material, and can thus be used without reference to considerations of quantity of material available.

Distribution of Cell Volumes

The measurement of the egg volume was the most precise datum available, and was computed, after determination of the diameter, from the formula,

$$V = 4/3\pi r^3.$$

Since volume is a cubic function of the radius, r,

$$dV/dr = 4\pi r^2,$$

and it may be noted that the difference in volume accruing from an error of 1 μ in the measurement of the diameter of a 74 μ egg is about 4 per cent; and that the radius is to be determined as accurately as possible.

A specially designed glass chamber, about 0.6 mm. deep, and with optically plane walls, was used to contain the eggs. After a suspension was pipetted into it, the open portion was covered over with a cover slip, and evaporation thus prevented. Egg diameters were measured with a filar ocular micrometer whose scale could be read to a fraction of a micron. Recalibrations of the ocular scale were frequent, to remain certain of its constancy. From each batch of eggs used, the diameters of about ten eggs were measured, and their volumes averaged, to secure a representative figure.

As regards the extent of dispersion of egg volumes, it can be said from inspection of Fig. 1 that the volumes of eggs from different females vary considerably. It is not feasible, then, to assume a diameter of 74 μ (a figure commonly employed) when the variation is so great. The curve was drawn from data on the volume and diameter of eggs counted and measured throughout the summer of 1934. The mode occurred at slightly less than 72 μ diameter, corresponding to a volume of about 193,000 μ^3. The extremes encountered in these measurements were diameters of 64 and 81 μ. Whether the considerably wider distribution and different mode for *Arbacia* eggs reported by Glaser (1914) is intrinsically a seasonal effect and may obtain at other breeding periods, remains to be determined. Not only is the absolute diameter less variable for individual females, but the degree of dispersion of diameters of cells from one animal is approximately constant, regardless of the mode. To determine how individual urchins compare in the variation of egg volumes, 9 eggs were selected at random from each of 14 animals, and the cell volumes measured. The total variation in egg volume was similar to that of Fig. 1, but the coefficient of variation ($v = 100\sigma/M$, σ = standard deviation) was approximately the same for the eggs from each urchin; the average coefficient of variation was 5.4 per cent.

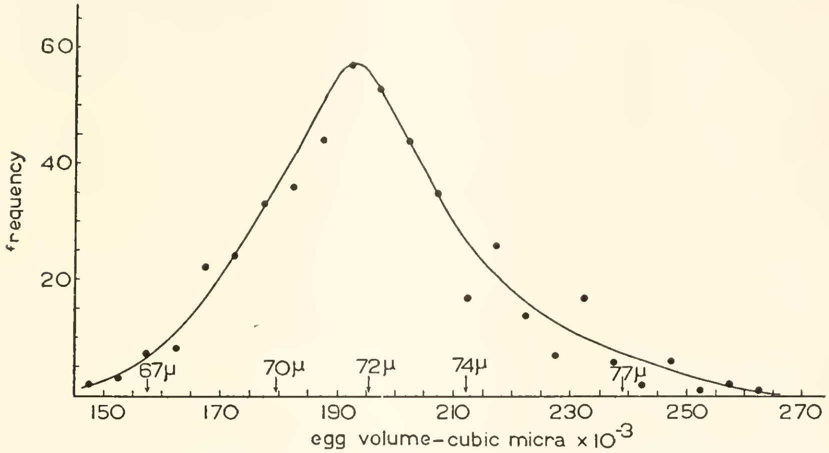

FIG. 1. Distribution of volumes of 467 eggs from 45 urchins selected at random during the season of 1934. The mode occurred at an egg diameter of slightly less than 72 micra.

Theoretical Considerations: the Stacking of Cells

If rigid spheres be stacked with their centers in line with each other, they occupy 52.36 per cent of the volume of the container; when nested fully, 74 per cent. These values hold regardless of the absolute diameter of the spheres, provided they are all of the same size. Thus, if *Arbacia* eggs were allowed to settle unhampered in a capillary tube, the volume read off should be 135.2 per cent of the true volume. They are prevented from actually nesting as close as this, under the influence of gravity alone, by the presence of the jelly, which envelops eggs freshly shed or removed from the ovaries. Owing to the presence of the jelly, whose average volume (from 15 cells) in one batch of freshly shed eggs was 1,123,000 μ^3 per egg, the eggs packed by gravity alone would occupy a volume equal to 9.9 times that of the eggs alone if the jelly envelope were to act as a rigid sphere. This ratio would be still higher (14.2 times) if we were to consider eggs which were taken after they had been allowed to remain in sea water for several hours, and whose jelly had consequently swollen to some degree.

Observations on the Jelly

Before they were counted, most of the eggs had large jelly envelopes; about 5 per cent had small coats of jelly. When centrifuged lightly (20 × gravity) in a test tube for one minute (to precipitate the eggs) the cells lose some of the outer looser layer of the jelly shell. In most of the batches of eggs used, even before handling, about 10 per cent of the eggs had little or no jelly. After pouring one batch of eggs back and forth between two tumblers, about nine times, about 50 per cent of the cells lost their jelly. Before sample drops were taken for counting, the eggs were thoroughly stirred in the tall Stender dish into which they had been poured originally. After about ten repeated resuspensions occasioned by withdrawal of samples for counting, about 50 per cent of the eggs were observed to have little or no jelly, and from the remainder the jelly was mostly sloughed off. The gradual reduction of the jelly envelope could be observed by examination of samples of the suspensions, between counts, in Chinese ink. To determine the amount of swelling which the jelly undergoes, the diameters of eggs and of jelly of cells freshly placed in a suspension of Chinese ink in sea water at 8:33 P.M. (urchin opened at 8:27 and eggs placed in dry Syracuse watch glass) were measured (8:35–8:44 P.M.). The average volume of the jelly of 15 cells was 1,123,400 μ^3 per cell. The same cells were measured again about two hours later (10:45–11:00 P.M.), and the average volume of the jelly was found to be 1,690,000 μ^3, or an increase, due to swelling, of about 50 per cent. When considered individually, the jelly of each cell, without exception, displayed the increase.

In centrifuging in dilute suspensions in wide tubes, and in an isosmotic and isopycnotic medium, the jelly is drawn over toward the centripetal end of the egg. When a column of eggs packed in a capillary tube was examined after centrifuging, the centripetal end of the column frequently showed a transparent region of varying length (1–1.5 mm.); this was taken to be jelly which had found its way to that region of the tube under the influence of centripetal force.

Comparison of Hemacytometers

Since the hemacytometer is an instrument originally designed for use with much smaller cells than *Arbacia* eggs and since, further, the diameter of the egg with its jelly usually greatly exceeds the depth of the 0.1-mm. slide, it was desirable to compare the latter instrument with one of twice its depth (0.2 mm.) and hence larger than the dimensions of the extracellular structures. For estimating the concentration of suspensions with hemacytometers, each batch of eggs was divided into three lots of the same concentration: *A*, for counting with hemacytometer of 0.1-mm. depth, *B*, for counting with 0.2-mm. hemacytometer, *C*, for centrifuging without having been subjected to preliminary counting, which entailed removal of the jelly in various degrees. As a matter of routine, some eggs were inseminated to determine their fertilizability. Only batches with a high percentage of fertilization (95 or more) were used. To remove large particles, the eggs were passed before counting and centrifuging through bolting silk, the size of whose mesh was approximately 175 μ on a side. In consequence of this treatment, about 10 per cent of the eggs were observed to have their jelly much reduced, or absent. The hemacytometers and cover slips were cleaned and dried thoroughly before each count was made. Before each sample was taken, the eggs were suspended uniformly, the suspension taken up quickly in a pipette, with the coverslip held in readiness by a pair of forceps, and a sample drop placed rapidly over the rulings, and the cover slid on immediately. The procedure usually resulted in a fairly even distribution of the cells. Care was taken to avoid irregularity in distribution of eggs in the hemacytometer. Only those drops were counted wherein the eggs appeared uniformly distributed after the cover glass was placed on the counting chamber. Counts on Lot *A* were made alternately with counts on Lot *B*. The 0.1 hemacytometer was ruled so as to contain four large squares (each 1 mm.^{2}) and the 0.2 instrument was ruled into 16 squares (1 mm.^{2}), hence more sample drops were counted, as a rule, with the shallower instrument. After counts were made by the above procedure, the concentration of Lot *A* was measured with the 0.2-mm. chamber, and commutatively, the 0.1-mm. chamber was used for Lot B. These latter measurements were fewer in number than those of the main series. The results, which are given in Table I, show for most counts, approximately a 12 per cent increase in the concentration when measured by the deeper slide. This is attributed to the influence of the jelly, and the action of the coverslip in tending to "squeeze out" eggs when placed over the drop on the shallower trough. In comparison with the centrifuge determinations, to be described presently, the results with hemacytometers were not found to be as reproducible. Averaging hemacytometer counts yielded a fair approximation to reproducible figures.

TABLE I

Comparison of concentration of egg suspensions as estimated by shallow hemacytometer (0.1 mm.) and by deeper hemacytometer (0.2 mm.).

Date	No. sample drops	Lot *A* 0.1 hcyt. *eggs per cc.*	No. sample drops	Lot *B* 0.2 hcyt. *eggs per cc.*	Difference in per cent *B/A*	No. sample drops	Count of Lot *A* with 0.2 hcyt.	No. sample drops	Count of Lot *B* with 0.1 hcyt.	Difference in per cent *A/B*
August 25....	9	205,000				5	220,200			
29....	9	391,500	6	394,600	+ 0.8	1	343,500	2	306,000	+12.2
30....	11	192,000	5	231,500	+20.6	2	192,300	2	200,000	− 3.8
September 3.	6	167,400	3	189,500	+13.2	2	251,800	1	158,000	+59.4
6.	9	237,000	3	265,200	+11.9	1	286,500	3	260,000	+10.2
10.	6	179,000	3	201,000	+12.3	2	251,000	4	210,300	+19.3
19.	10	178,500	6	174,400	−2.35					

Centrifuge Tubes and Centrifuges

Centrifuge tubes of the type diagrammed in Fig. 2, with a capillary length which varied from 6 to 7 cm., were used. The tubes were sealed off at one end, to avoid possible loss of a slight amount of fluid at high centrifugal forces, as may occur in the use of tubes of conventional hematocrit design, with open ends. Inasmuch as sealing the tubes at one end in this way entails the formation of a meniscus, the closed portion of the capillary was blown out very slightly in an attempt to compensate. The error involved is about 0.5 mm., and in a column of 6 cm. this is a volumetric error of only a fraction of 1 per cent. Krueger (1930) placed a small drop of mercury in the bottom of the tube to secure a more nearly plane column end of cells. Hastings (1921) described the use of a graduated thermometer capillary as a hematocrit, designed by E. L. Scott. Ungraduated capillary tubes of bore 0.8 mm. and 1.6 mm. were used in the present experiments, and gave comparable results at 2,700 and 7,700 × gravity. The capillaries were calibrated for volume per unit length by filling with mercury to various lengths, and weighing precisely.

The cells were mixed and suspended by gentle agitation. A measured sample was pipetted up, and the suspension of cells allowed to flow quickly from the pipette (to avoid settling) into the wide tubing spliced to the capillary. It is not necessary initially to fill the capillary before centrifuging for the air is displaced by the suspension during centrifuging, and the cells and the liquid fill the capillary in a continuous column. The capillary can be cleaned out easily by inserting into it a capillary pipette attached to a tap water suction pump, and immersing the mouth of the tube under water.

The length of the column was measured by placing the tube on a mirror (to avoid parallax) parallel to a steel rule graduated into millimeters. In this way the length of the column could be estimated to about 0.2 mm. The agreement between duplicate determinations of total egg volume by centrifuging was good, and, in the writer's hands, the centrifuge gave less variable results than the hemacytometer. The chief variable in readings of this nature seems to arise from the inhomogeneity in the distribution of the eggs in the suspension from which samples are pipetted. Seventeen duplicate determinations of volume by centrifuging at 2,700 × gravity showed an average agreement of 2.4 per cent, which was much better than duplicate determinations by successive hemacytometer counts. The centrifuge used was a type generally available (International, size 2, head 325); the centrifugal force attainable with this machine was 2,700 × gravity, and large tubes of any size could be accomodated. The centrifuge method may be used in two ways: (*a*) for ultimate packing; or (*b*) where a high speed centrifuge allowing nearly complete packing is not available, the introduction of a conversion factor, when the relative centrifugal force and duration of centrifuging are maintained constant.

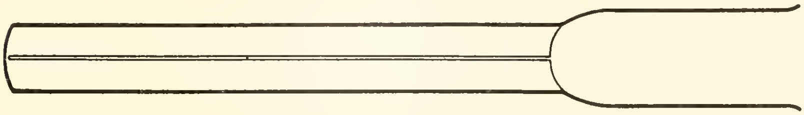

FIG. 2. Diagram of centrifuge tube, to show capillary and cup for receiving cell suspension. Used for centrifuging at 2,700 times gravity.

General Effects of Centrifuging Cells

The first run was made on material whose measurements are given in Fig. 3. It is clear that for any given mass of cells the degree of packing of the cells depends upon two variables, viz., the duration of centrifuging, and the centrifugal force. Each of the component curves becomes asymptotic to a given capillary volume at a particular centrifugal force. In this sense, perhaps, constant volumes are not obtained, but if centrifuged long enough, the volume is constant within the errors of measurement. At an early stage of packing, it is relatively easy, at low centrifugal forces, to compress the egg suspension to a smaller volume. At any specified centrifugal force a final degree of packing of cells is attained after a minimum amount of centrifuging. In this particular run, the total "centrifuge volume" of eggs contained in 1 cc. of suspension (0.0559 cc.), as estimated from the volume of the capillary occupied by the packed eggs, was about 10.6 per cent higher than that computed from the hemacytometer (0.0506 cc.). The ratio of 1.8 to 1 of cell volume by centrifuge and hemacytometer, reported by Gerard and Rubinstein (1934), is understandable when it is noted that low centrifugal forces (400 and 750 times gravity) were used, and relatively short periods of centrifuging (1 to 10 minutes). The further analysis of

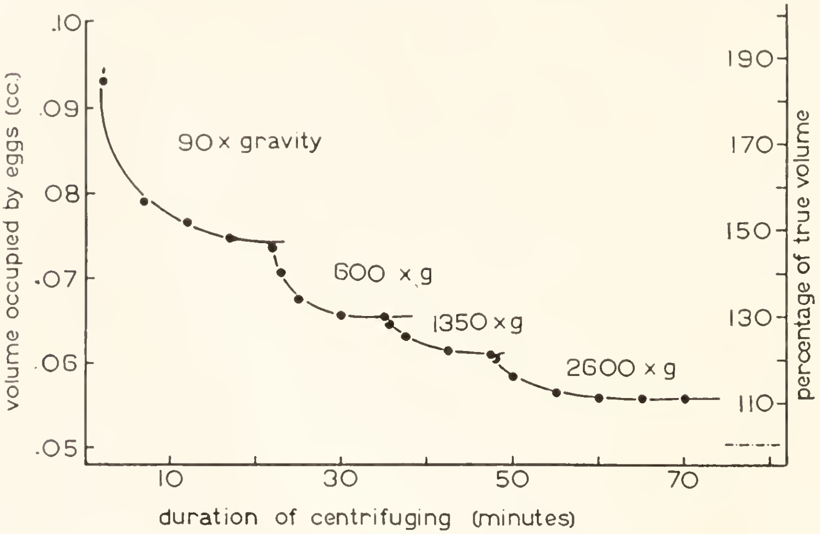

FIG. 3. Effect of duration of centrifuging, and centrifugal force on the volume occupied by a suspension of cells in a capillary. All the data were obtained from a single suspension of cells centrifuged in the same tube. The volumes were measured at various times after starting the centrifuge, and when an approximately constant value had been attained, the centrifugal force was raised. At any given centrifugal force the volume decreases rapidly at first and then approaches asymptotically a constant value. The dot-dash line near the bottom of Figs. 3 and 4 represents in each case the "true volume" as determined from hemacytometer measurements. It is to be emphasized that these curves were not intended to be used as a basis for standard conversion factors at centrifugal forces lower than 2,700 times gravity; they are presented largely to represent the nature of the changes which occur. Figs. 3, 4, and 5 were drawn from the same set of data.

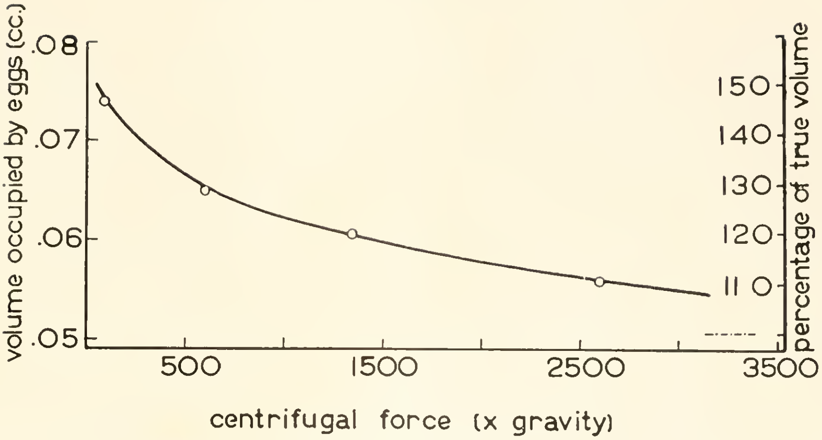

FIG. 4. Relation between centrifugal force and final (asymptotic) value.

this set of data is given in the remaining figures. In Fig. 4, the asymptotic values are plotted for each centrifugal force, and from Fig. 5 it appears that for progressively equal decrements of the excess volume, as read from the capillary length, it was required to increase the centrifugal force, not in equally proportional increments, but rather in cubes of these increments. The expression for this rectilinear relation,

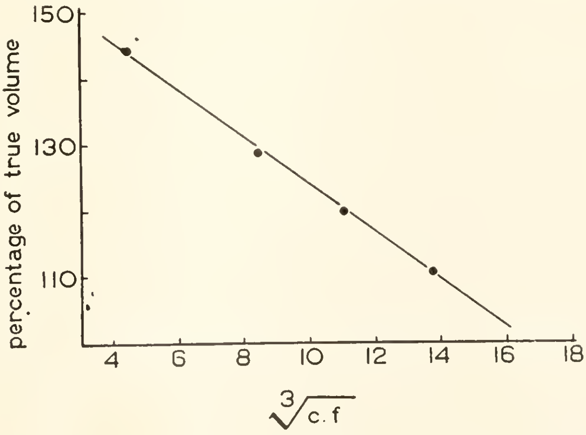

FIG. 5. To show the nature of the dependence of the asymptotic volume occupied by *Arbacia* eggs in a capillary, upon centrifugal force (C. F.).

$$P = -3.68\,(C.F.)^{1/3} + 160,$$

where P is the percentage of the true volume, and $(C.F.)$ is the centrifugal force (relative to gravity), holds with good approximation for this

Table II

Comparison of total volume of unfertilized eggs as determined by hemacytometers, and by centrifuging in capillary tubes at 2,700 × gravity for 20 minutes. Below, data on fertilized eggs, using volume of unfertilized eggs as basis of comparison.

Date	Volume by hemacytometers	Volume by centrifuge (2,700 × g)	Difference
	cc.	*cc.*	*per cent*
August 25	.0766	.0816	+ 6.5
	.0506	.0537	+ 6.2
29	.0880	.0787	−10.5
	.0440	.0416	− 5.5
	.0440	.0404	− 8.1
	.0880	.0752	−14.6
	.0880	.0744	−15.5
30	.0434	.0424	− 2.4
	.0434	.0452	+ 4.1
	.0656	.0647	− 1.4
	.0434	.0436	+ 0.6
September 3	.0371	.0457	+23.2
	.0742	.0906	+22.1
	.0742	.0925	+24.5
6	.1068	.1242	+16.3
	.0534	.0624	+16.9
	.03204	.03687	+15.1
	.1068	.1238	+16.0
10	.1317	.1289	− 2.1
	.03512	.0373	+ 6.2
19	.0715	.0612	−14.4
	.1072	.0940	−12.3
	.1072	.09514	−11.3
*	.0867	.0940	+ 8.4
*	.0578	.0612	+ 6.0
*	.0867	.09514	+ 9.8
*20	.10632	.1042	− 2.0
*	.0709	.0680	− 4.0
*	.10632	.1028	− 3.4
*	.1122	.1204	+ 7.3
*	.1122	.1185	+ 5.6
Fertilized Eggs			
August 29	.088	.0763	−13.3
	.088	.0794	− 9.7
30	.0651	.0739	+13.7
September 3	.03902	.04836	+23.8
	.07804	.1000	+28.2

* Volume determined in these cases by dilution method, not hemacytometer.

series of measurements, and affords a satisfactory conception of the nature of the dependence of the "centrifuge volume" upon the centrifugal force. For $P = 100$, i.e., the volume by centrifuge is equal to the true volume (or that calculated by hemacytometer), a centrifugal force of 4,330 × gravity is necessary, as calculated from this empirical formula, assuming that a linear extrapolation can be made. For other sets of data, the curve as a whole would be shifted slightly to the left or to the right, and the extrapolated value would change accordingly.

Results: Agreement between Hemacytometer and Centrifuge Volumes

In Table II, where the data comparing centrifuge and hemacytometer volumes are summarized, a minus sign before the differences indicates that the volume as estimated by centrifuge at the given centrifugal force was less than that as measured by hemacytometer, by the given percentage; and conversely, the plus signs indicate a greater volume by centrifuge as compared to hemacytometer. The values obtained from the two hemacytometers were averaged, and used as a standard basis for comparison with the volumes obtained by centrifuging. A negative value in the comparison of centrifuge with slide would seem to indicate that the slide estimate was too high, or may arise from differences in sampling due to inhomogeneity. The values in the table may be summarized by noting that of 29 determinations at 2,700 × gravity, 15 showed an average of 12 per cent greater volume than that estimated by hemacytometer, and 14 showed an average of 7.7 per cent less than that evaluated by the hemacytometers. Thus the two methods (hemacytometer, 0.1 and 0.2 mm. depths, and centrifuging at 2,700 × gravity for 20 minutes) agreed to within approximately 10 per cent, which may be taken as an average for all the experiments. A few experiments on fertilized eggs are also included in the table.

It would seem that the jelly of the *Arbacia* egg (which, as in these experiments, has been exposed to sea water for approximately one to three hours) is easily displaced or squeezed out by the eggs in centrifuging, and hence does not exert a significant effect in these experiments, for wherever comparisons of Lots *A*, *B*, and *C* were made, where there was no partial reduction of jelly in *C* since it was not mixed and resuspended repeatedly for counting, the measurements of volume by centrifuging resulting from use of portions from each of the lots were identical within experimental error.

Dilution Method

By the use of a procedure suggested by Dr. A. K. Parpart, a check on the two methods described above was made by an independent dilution method. Small portions of egg suspension were diluted to varying degrees in large volumes of sea water, placed in glass-stoppered bottles, and inverted several times to suspend the eggs uniformly. Then a capillary tube, slightly larger than 1 mm. in bore, and marked off at a length of about 9 cm. (total volume to mark, 0.168 cc.) was quickly immersed and withdrawn, and the total number of eggs contained in the capillary counted directly by examination with a binocular dissecting microscope. Knowing the dilution and the number of cells in the capillary, the original number of cells in the concentrated sample could be calculated. A protocol of some results obtained by this method, which was not studied as thoroughly as the hemacytometer method, is given below. The egg concentration estimated by this dilution method gave figures about 15 per cent lower than those estimated by the hemacytometer.

No. samples counted	Av. concentration by hcytr.	Dilution method	No. eggs per cc.	Centr. tube	$\frac{\text{Centr. vol.}}{\text{Hcyt. vol.}}$	$\frac{\text{Centr. vol.}}{\text{Dilution vol.}}$
10	176,500 cells/cc.	5 cc. : 140	141,520	B	$\frac{.06115}{.07148}=85.6\%$	$\frac{.06115}{.0578}=106\%$
		5 cc. : 500	148,570	D	$\frac{.0940}{.1072}=87.7\%$	$\frac{.0940}{.0867}=108.4\%$
		5 cc. : 1,000	138,700	C	$\frac{.09514}{.1072}=88.7\%$	$\frac{.09514}{.0867}=109.8\%$

In another series of determinations, the dilution and centrifuge methods only were compared, and gave very good agreement. The data are given below; all figures were obtained from measurements on a single suspension.

Comparison of Dilution and Centrifuge Method

No. samples counted	Dilution	Average concentration	Tube	$\frac{\text{Centrif. vol.}}{\text{Dilution vol.}}$
4	2 cc. : 140 cc. s.w.	185,000 cells/cc.	B	$\frac{.068}{.0709}=0.96$
8	2 cc. : 500 " "	184,700 " "	C	$\frac{.1042}{.1063}=0.98$
6	2 cc. : 1,000 " "	167,400 " "	D	$\frac{.1028}{.1063}=0.97$
7	6 cc. : 1,000 " "	177,700 " "		

Data on the Absolute Respiratory Rate of Unfertilized Arbacia Eggs

It may be of interest to note that measurements of the respiration (9/20/34) of eggs taken from urchins kept in laboratory aquaria for a period of about four weeks, with Warburg manometers, at 25.9° C., showed a Q_{O_2} (cu. mm. oxygen per 10 cu. mm. eggs per hour) of 1.03 for unfertilized eggs, and 3.04 for fertilized eggs (average of three determinations of each). The absolute volume of eggs was determined by combining the values obtained by centrifuge and dilution methods. Reduced to 21° C. by applying a Q_{10} of 4.1 for unfertilized eggs (Rubinstein and Gerard, 1934), the Q_{O_2} becomes at that temperature 0.52. More representative is the average of a series of measurements carried out from July to September, 1934, with Warburg and Fenn respirometers, at 25.9° C., and using the hemacytometer method for estimating egg volume (reported in detail elsewhere: Shapiro (1935)). The Q_{O_2} for unfertilized eggs was 1.5. When reduced to 21° C. this value becomes 0.75.

Development of Eggs after Centrifugal Compression in a Capillary

After centrifuging, unfertilized eggs were removed from the capillary and examined microscopically. They were found to be well stratified in the usual manner, drawn out into cylinders, with corners somewhat truncated, a configuration lending itself to more intimate packing of the cells. One batch of cells which had been centrifuged 20 minutes at 2,700 × gravity was left in the capillary 24 minutes in the packed condition, and then removed and inseminated. They retained their elongated form after fertilization, and proceeded to cleave and develop. The pigment remained at one pole of the cell. This was demonstrable repeatedly and indicates that cells can easily survive these experimentally induced conditions. The toughness of the fertilization membrane prevents eggs from elongating, when centrifuged after insemination. The fertilized eggs, after packing, were poorly stratified and in some cases practically unstratified, and polygonal in outline, and retained the fertilization membrane, which usually enveloped the cell closely. They tended to resume the spherical configuration when replaced in sea water after centrifuging. On several occasions it was found that cells fertilized and then packed in a capillary by centrifuging at 7,700 × gravity for 20 minutes would upon removal from the tube proceed to develop as far as free-swimming plutei. Whether water is actually abstracted from the cell as a result of centrifugal compression, is an open question. R. S. Lillie has observed (1918) that fertilized cells, which have shrunken in hypertonic sea water faster than resting egg cells, appear denser than unfertilized eggs exposed for the same period of time in that they sink faster to the bottom of the container.

Related Experiments at Higher Centrifugal Force

Numerous experiments were made on living eggs swollen and shrunken, and dead cells of various volumes fixed in formalin and centrifuged at 7,700 × gravity to determine volume. The technique involved the use of large volumes of fluids and subsequent decantation of the supernatant fluid, following light centrifuging, in order to get the cells into the small tubes used, which entailed their addition in several small portions, with centrifuging between additions. This procedure was necessary because of the short length of the slots in the high speed centrifuge head, and the use of a long capillary column led to the sacrifice of the wide tube at the upper end, which might receive all the cells at once. The cells were added in small quantities, and the supernatant fluid removed after each preliminary centrifuging to make way for the rest of the cells. A slight (but indeterminate) loss of cells occurred owing to the adhesion of some of the material to the walls of the large test tubes in which they were kept during swelling or shrinking. Thus it was difficult for these reasons to demonstrate the adequacy of the centrifuge method for such cells. Data for normal, living unfertilized and fertilized eggs centrifuged for 20 minutes at 7,700 × gravity were also obtained, and may be summarized by stating that of 15 determinations, 6 showed an average volume 10.6 per cent less and 9 an average volume 12.8 per cent greater than that estimated by hemacytometers. It is of interest to add that when eggs are placed in successive small lots in these small tubes, they appear rhythmically stratified, i.e. each lot shows a light brown layer at the centripetal end.

SUMMARY

Observations of the aggregate volume of *Arbacia* eggs, when centrifuged in capillary tubes, as a function of centrifugal force, and of duration of centrifuging were made. Data on the comparisons of such total cell volume contained in suspensions of *Arbacia* eggs as evaluated by three methods (counting in hemacytometers, centrifuging at 2,700 and 7,700 × gravity, and direct counts of diluted suspensions) are given. It is submitted that the centrifuge method is reliable, to within approximately 10 per cent, for estimation of the total volume of cells in a suspension of unfertilized eggs of *Arbacia punctulata* in sea water provided that it be used with the necessary force and duration for sufficient packing of the cells.

Cells so centrifuged are in the living state, and remain viable, for upon removal from the capillary, they will proceed to cleave and to undergo considerable embryological development.

LITERATURE CITED

Gerard, R. W., and B. B. Rubinstein, 1934. A Note on the Respiration of Arbacia eggs. *Jour. Gen. Physiol.*, **17**: 375.

Glaser, O., 1914. The Change in Volume of Arbacia and Asterias Eggs at Fertilization. *Biol. Bull.*, **26**: 84.

Harvey, E. N., 1932. Physical and Chemical Constants of the Egg of the Sea-urchin, Arbacia punctulata. *Biol. Bull.*, **62**: 141.

Hastings, A. B., 1921. The Physiology of Fatigue. Physico-chemical manifestations of fatigue in the blood. *Public Health Bull.* 117, p. 16.

Krueger, A. P., 1930. A Method for the Quantitative Estimation of Bacteria in Suspensions. *Jour. Gen. Physiol.*, **13**: 553.

Lillie, R. S., 1918. The Increase of Permeability to Water in Fertilized Sea-urchin Eggs and the Influence of Cyanide and Anaesthetics upon this Change. *Am. Jour. Physiol.*, **45**: 406.

Rubinstein, B. B., and R. W. Gerard, 1934. Fertilization and the Temperature Coefficients of Oxygen Consumption in Eggs of Arbacia punctulata. *Jour. Gen. Physiol.*, **17**: 677.

Shapiro, H., 1935. The Respiration of Fragments Obtained by Centrifuging the Egg of the Sea-urchin, Arbacia punctulata. *Jour. Cell. Comp. Physiol.* **6**: 101.

Whitaker, D. M., 1933. On the Rate of Oxygen Consumption by Fertilized and Unfertilized Eggs. IV. Chaetopterus and Arbacia punctulata. *Jour. Gen. Physiol.*, **16**: 475.

AN ANALYSIS OF THE ACTION OF LITHIUM ON SEA URCHIN DEVELOPMENT

JOHN RUNNSTRÖM[1]

(*From the Marine Biological Laboratory, Woods Hole, Mass.*)

The fact that lithium added to sea water profoundly modifies the sea urchin development was discovered by Herbst (1892, 1893, 1895). The most important change involved is the enlargement of the entomesoderm at the expense of the ectoderm. It is important to stress that this shift in the relative amounts of ectoderm and entoderm is not occasioned by any destruction or throwing off of ectoderm material during the early development but by some kind of a change in the determination of the egg. This would mean that the potentialities for producing ecto- and entoderm are so affected by lithium as to favor those which are concerned in the production of entoderm. While Herbst (1893) and Spek (1918) in their interpretation of the "lithium" development directed their main attention towards the vegetative part, MacArthur (1924) and Runnström (1928, 1929, 1933) have emphasized the effect of lithium on the animal part of the larva. All our experimental evidence indicates that the susceptibility to the action of lithium is highest at the animal pole and decreases gradually.

The writer (1929, 1933) has developed the conception that in the sea urchin egg, there exist two opposite gradient systems, one preponderating at the animal, the other at the vegetative pole, as illustrated diagrammatically in Fig. 1. According to this hypothesis, the determination along the egg axis would depend upon the balancing effect between the animal and the vegetative gradient system. The lithium type of development would therefore be due to a shift in the normal balance, as illustrated by Diagram *B* in the figure. The animal gradient system weakens and the vegetative gradient system gains the ascendancy. The ring of skeleton-forming mesenchyme cells appears further towards the animal pole and the boundary between ectoderm and endoderm moves closer to the animal pole of the egg. In extreme cases this condition may be carried to the extent that the entire embryo is transformed into entomesoderm.

Under other conditions the opposite effect is known to occur. For example, by exposing the eggs to certain agents, the vegetative gradient system weakens in comparison to the animal gradient system, cf. Diagram *C* in the figure. As a consequence of the resulting unbalance, the animal region becomes increased (Herbst, 1904; Lindahl, 1933).

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[1] Fellow, Rockefeller Foundation, 1933–34.

The theory briefly given here is substantiated also by numerous isolation and transplantation experiments carried out by Hörstadius (1935). Only one of these experiments may be mentioned. In the 64-cell stage Hörstadius isolated that one of the two cell rings, originating by the division of the macromeres, which lies at the more vegetative end. This cellular material in normal development gives rise to entoderm only. The isolated ring developed into a larva with both entoderm and ectoderm. Evidently by a regulative process, an almost normal balance had become established in the isolated cell ring. By implantation into this cell ring of one, two, or four macromeres (the most vegetative material), this balance was disturbed and larvæ developed which most strikingly resembled lithium-treated larvæ. Hence an increase in amount of micromere material produced the same effect as an increase in lithium concentration.

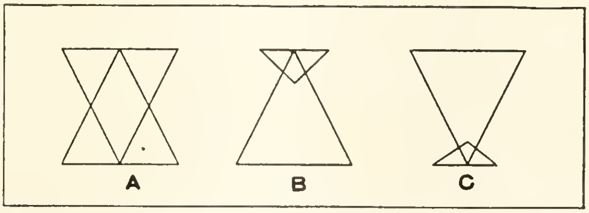


Fig. 1. Diagrammatic representation of the gradient systems in the sea-urchin egg.

The mode of action of lithium has been assumed by Spek (1918) to be a precipitating and swelling effect on the surface of the vegetative cells. This, according to Spek, would account for the " exogastrulation " which is one of the most characteristic effects of treatment with stronger concentrations of lithium. Runnström believes, on the other hand, that such a surface effect is secondary to a more important internal effect and he has experimental evidence to show that lithium penetrates the cells. With dark ground illumination the internal structure of the cells of the " lithium " larva has a coarsened appearance. He assumes that some action of the lithium which is related to this structural change has an effect on the determination and differentiation of the animal pole greater than on the vegetative pole.

Runnström (1933) found that lithium, in concentrations which in themselves are too low to affect development, can be made very active if the eggs, during exposure, are kept in an atmosphere of carbon monoxide containing 5 per cent oxygen. This effect of carbon monoxide is abolished in strong light. The same, as is well known, holds true from Warburg's work (1932) on the effect of carbon monoxide on respiration. This evidence, that the effects of lithium and carbon monoxide are additive, suggests that these two agencies have a similar action on the developing egg. In support of this is the finding of Lindahl (1933, 1934) that lithium has an inhibiting action on the respiration of the egg. Warburg (1915) has shown that a steady increase of respiration takes place during the early development of the sea urchin egg. It is only this increasing fraction of the respiration which is affected by lithium according to Lindahl. This fraction has a special character and may be identical with the oxidation of breakdown products of certain carbohydrates (Lindahl, 1933, 1934, 1935).

During my sojourn at the Marine Biological Laboratory at Woods Hole during the summer of 1934, I had the opportunity of securing the assistance of Professor Robert Chambers in a microdissection study on the physical properties of the eggs of the sand dollar, *Echinarachnius parma,* treated with lithium.

The eggs of this species proved to be extremely sensitive to the action of lithium. A solution of 100 cc. sea water and 5 cc. of 2.6 per cent LiCl killed them in the early stages of segmentation, while the eggs of *Arbacia punctulata* (similar to the European *Paracentrotus lividus*) segment and develop in this solution for more than twenty hours.

It was found that the sand dollar eggs will develop in a weaker solution, for example: eggs immediately after fertilization were placed in 2 cc. of 2.6 per cent LiCl to 100 cc. sea water and, after a sojourn of 20 hours, were transferred to normal sea water. The larvæ exhibited the typical "lithium" effect, namely, a retarded development, a shift of the position of the mesenchyme cell ring and an enlargement of the entodermal region followed by exogastrulation and differentiation of ectoderm and skeleton.

It was found that the physical state of the cells of the larvæ which had been exposed to lithium in sea water was demonstrably different from that of controls developed in sea water alone. The blastulæ were pierced and held in place in the hanging drop (in the microdissecting chamber) by means of one micro-needle and the epithelial wall was torn by means of another needle. In this way strands of the epithelial cells could be stretched. The cells of the controls, after being torn apart and released, quickly became spherical. On the contrary, the cells from the "lithium" blastulæ remained distorted and spindle-shaped. The difference was striking and left no doubt that a profound change in the state of the cytoplasm had taken place as a consequence of the action of the lithium.

It was also found that the hyaline plasma layer which invests the larva is softened and rendered far more pliable when treated with lithium than the layer investing the untreated larva. This fact nullifies any assumption that an increased resistance to the swelling of the blastocœl and the enlargement of the blastula might have been due to a stiffened hyaline plasma layer.

A further observation on control larvæ may be mentioned in this connection. It is easy to observe, in the late blastula or the early gastrula stage, that the hyaline plasma layer presents many folds on the surface of the most vegetative part of the larva. A microneedle could be inserted into this part of the hyaline layer. When the layer was stretched the folds disappeared and a cone-like process was formed. Evidently the folds are formed when the form of the underlying cells changes preparatory to the invagination of cells during gastrulation. The later fate of these folded parts of the hyaline layer is difficult to follow but it is probable that an unfolding takes place when the entoderm cells subsequently enlarge their surface. No studies were made on the presence of folds in the hyaline layer in lithium-treated larvæ, but the folding of the hyaline layer occasionally was observed also in "lithium" larvæ. This speaks also against a stiffening of the hyaline plasma layer under the action of lithium.

The hyaline plasma layer can still be demonstrated even in the normal pluteus. A striking method is to leave the larva in a dish infested with certain Protozoa. These penetrate the dead larva and consume the cellular portion, leaving intact only the skeleton and an investing, fairly soft membrane.

During the development of the larva a considerable growth of the hyaline layer must also take place. This growth must be due to a secreting activity of the cells which normally are kept together chiefly by the hyaline layer. Herbst (1900) has discussed the factors involved in the coherence of the epithelial cells of the sea urchin larva. He considered the action of the hyaline layer and also a second factor, which according to Herbst is more important, and which keeps the cells together even when the hyaline layer has been removed. It seems probable, however, that this second factor is more pronounced only in larvæ which have been submitted previously to a treatment with calcium-free sea water.

During the early differentiation of the sea urchin larva the change in shape of the cells plays an important rôle. Already in the blastula stage a flattening of the cells has begun to take place. In the pluteus most of the aboral part of the ectoderm is formed by a flat epithelium. The indications are that the hyaline plasma layer plays only a subordinate rôle in changing the shape of the cells during development. A retardation in the flattening of the blastula cells is very characteristic for the lithium type of development. The observations made in the course of this work do not favor the idea that the retardation is due to the changed qualities of the hyaline layer. Probably retardation is caused by a " lithium " effect on the internal material of the cells.

The pronounced reaction of *Echinarachnius* eggs to LiCl in comparison with their reaction to chlorides of sodium and potassium are shown in the following experiment. Eggs, recently fertilized, were immersed in solutions of the three chlorides in concentrations approximately isotonic with sea water. In a KCl solution the eggs survived for several hours and underwent cleavage. In NaCl the survival was more limited, for, in the course of an hour, a certain percentage of the eggs underwent cytolysis and the rest at varying times somewhat later. In LiCl all the eggs were destroyed within 10–15 minutes. The surface of the egg burst and a part of the contents flowed into the space under the fertilization membrane while the remainder of the egg shrank somewhat into a clear cytolyzed mass in marked difference to the dark cytolized eggs in NaCl.

The experiments reported in the preceding pages tend to show that lithium has a direct influence on the structure of the cytoplasm. But the possibility is not excluded that the action of lithium, in the range of concentrations producing typical " lithium development," is more indirect. This is rendered probable by the following observations. Eggs of *Echinarachnius* were transferred very soon after fertilization into a solution of 100 cc. sea water containing 5 cc. of 2.6 per cent LiCl and into a similar solution containing 0.5 cc. of 0.1 per cent pyocyanine. In the lithium-sea water the eggs were killed in the two-celled stage but in the lithium-sea water plus pyocyanine a development to the blastula stage took place. In the control the development was normal. This experiment was repeated and varied as to the lithium concentration. It was always found that the addition of pyocyanine caused a marked improvement on the development in lithium-sea water. In a few experiments it was also found that the addition of methylene blue somewhat improves the development in lithium-sea water. From experiments carried out by Friedheim (1931), it is well known that pyocyanine has a promoting action on the respiration of several kinds of cells. Runnström (1935) has shown that this is true also for the fertilized sea-urchin egg. It can thus safely be inferred that pyocyanine acts by inducing oxidation processes which are able to replace, to a certain degree, those suppressed by lithium.

Runnström (1928) found that the addition of KCl to the lithium-sea water in somewhat more than equimolecular concentration to that of the lithium removes the modifying influence of the lithium on morphogenesis. Lindahl (1933) showed that this addition of KCl also removes the inhibitory action of lithium on respiration. From unpublished experiments of mine on *Arbacia* eggs I have found that potassium is more efficient than pyocyanine on the restitution process.

From the facts reported and discussed above it follows that one has not only to consider a general effect of lithium on the physical state of structure but also a specific effect on the metabolism of the sea urchin egg and embryo. This specific effect may possibly be the primary one. These aspects will be more fully discussed in a paper by Lindahl (1935).

Summary

The eggs of *Echinarachnius parma* are very sensitive to the action of lithium added to sea water.

A concentration of lithium was used which produces the typical "lithium development." The epithelial cells of normal and of lithium-treated blastulæ were stretched by microneedles and released. In normal larvæ the cells round up after release; in the lithium-treated larvæ they remain deformed. The hyaline plasma layer does not stiffen in the lithium-sea water and is not necessary for the flattening of the cells which takes place during the development. The presence of folds of the hyaline plasma layer at the vegetative pole in the late blastula and the early gastrula stage is described.

Pyocyanine, added to the lithium-sea water, counteracts the effect of the lithium and improves development. This and other facts indicate that lithium does not only exert an influence on the structure of the protoplasm but also on the respiration.

I wish to express my sincere thanks to Professor Robert Chambers for his generous assistance in carrying out the micro-dissection work reported in this paper and for correcting and criticizing the manuscript.

LITERATURE

Friedheim, E., 1931. *Jour. Exper. Med.*, **54**: 207.
Herbst, C., 1892. *Zeitschr. wiss. Zool.*, **55**: 446.
Herbst, C., 1893. *Mitth. Zool. Station Neapel*, **11**: 136.
Herbst, C., 1895. *Arch. entw.-mech. Org.*, **2**: 455.
Herbst, C., 1900. *Arch. entw.-mech. Org.*, **9**: 424.
Herbst, C., 1904. *Arch. entw.-mech. Org.*, **17**: 306.
Hörstadius, S., 1935. *Comm. Stazione Zool. Napoli*, **14**: 251.
Lindahl, P. E., 1933. *Arch. entw.-mech. Org.*, **128**: 661.
Lindahl, P. E., 1934. *Naturwiss.*, **22**: 105.
Lindahl, P. E., 1935. *Acta Zoologica*, in press.

MacArthur, J. W., 1924. *Biol. Bull.*, **46**: 60.
Runnström, J., 1928. *Acta Zoologica*, **9**: 365.
Runnström, J., 1929. *Arch. entw.-mech. Org.*, **117**: 123.
Runnström, J., 1933. *Arch. entw.-mech. Org.*, **129**: 442.
Runnström, J., 1935. *Biol. Bull.*, **68**: 327.
Spek, J., 1918. *Kolloidchem. Beih.*, **9**: 259.
Warburg, O., 1915. *Arch. ges. Physiol.*, **160**: 324.
Warburg, O., 1932. *Zeitschr. angw. Chemie*, **45**: 1. (Review.)

THE LUNGS OF THE MANATEE (TRICHECHUS LATIROSTRIS) COMPARED WITH THOSE OF OTHER AQUATIC MAMMALS

GEORGE B. WISLOCKI

(From the Department of Anatomy, Harvard Medical School, Boston, Massachusetts)

In 1929 I reported the histological structure of the lungs of the porpoise (*Tursiops truncatus*) and discussed the findings in relation to the aquatic mode of life of whales. The present report concerns the finer structure of the lungs of another aquatic mammal, the manatee (*Trichechus latirostris*), belonging to the order of Sirenia or sea cows. Concerning the histology of the sirenian lungs, the only previous account is a description by Pick (1907) of the lungs of *Halicore dugong*, which omits a very complete account of its finer histology.

The ultimate aim of these studies is to obtain a knowledge of the modification of the respiratory tract in mammals which have adopted an aquatic mode of life. To date, in addition to the observations of Pick on the dugong, fuller accounts exist for certain Cetacea, namely, three of the porpoises, *Delphinus* (Fiebiger, 1916, and Lacoste and Baudrimont, 1926), *Tursiops truncatus*, the bottle-nosed porpoise (Wislocki, 1929), and *Phocaena communis*, the harbour porpoise (Lacoste and Baudrimont, 1933). The present account of the lungs of the manatee adds the new world representative of the order Sirenia to the observations of Pick on the old world form, the dugong, and makes it possible to undertake a wider comparison of the Sirenia with the porpoises of the order Cetacea.

Anticipating the results of our examination of *Trichechus*, it may be said that they resemble rather closely the account given for the dugong by Pick. Turning to the Cetacea, comparison of *Delphinus* and *Tursiops* (both Delphininae) has shown that these two species agree with one another in almost every detail. On the other hand, the smaller harbour porpoise, *Phocaena*, representing the Phocaeninae, exhibits, according to Lacoste and Baudrimont, a number of major differences from the Delphininae. The latter finding suggests that there may be a number of modes of specialization of the lungs within the order Cetacea. Finally, a comparison of the two types of Sirenia (which in regard to lung structure show a close resemblance) with the two types encountered thus far in the porpoises, should allow us in a beginning measure to discriminate between the structural changes which are generalized aquatic adaptations and those which represent specializations within the narrower confines of an order or family of mammals.

Material

The material on which the present description is based consists of the lungs of an adult manatee, freshly fixed *in toto* in 10 per cent formalin. Sections of the lungs were studied after staining with haematoxylin and eosin, Mallory's connective tissue stain, and Weigert's resorcin fuchsin. The preservation of the tissues, although by no means perfect, allows a considerable amount of the histology of the lungs to be ascertained, as the accompanying photographs of the sections illustrate.

Description of Material

The description of the material will be kept as brief as possible, the appended illustrations offering the best means of presenting the findings. No presentation is deemed necessary of the naked-eye topography of the lungs, because on reading over Pick's rather complete account of the gross appearance of the lungs of the dugong and comparing it with my specimen of the manatee, I have come across no salient differences in anatomical arrangement. It will suffice, therefore, to present merely one photograph of the gross appearance of the specimen, namely, a transverse section or rather slab of tissue obtained by cutting in half one of the two elongated, flattened lobes which characterize the sirenian lungs (Fig. 1). To the right of the figure on the ventral border the main bronchus can be seen accompanied by the pulmonary artery (*p. a.*) and vein (*v. p.*).

Explanation of Plate I

Fig. 1. A transverse slab of tissue from the right lung of a manatee. Notice the main bronchus (*br. 1*) on the ventral surface. Nearby are the pulmonary artery (*p. a.*) and vein (*p. v.*), besides a subsidiary bronchus (*br. 2*). × .34.

Fig. 2. A section of the slab shown in the preceding figure covering about two square centimeters, showing the tremendous size of the alveolar sacs and the heavy septa bounding them. × 2¾.

Fig. 3. A section of human lung, at the same magnification as the preceding figure, to show the tremendous differences between the two in size of air sacs, thickness of the pleura, and the density of the stroma of the lungs. × 2¾.

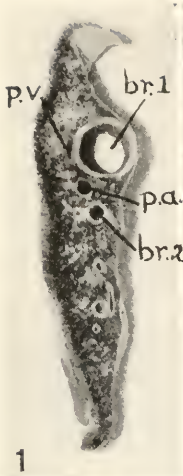
Fig. 4. A photograph of the pleura of the manatee stained for elastic tissue to show: (1) the outer collagenous layer; (2) the dense lamina of elastic tissue in the middle; and (3) on the inner side the vascular layer. × 30.6.

Fig. 5. A section of a cat's lung for comparison with the manatee lung (Fig. 2) and the human lung (Fig. 3), all at the same magnification. × 2¾.

Fig. 6. A photograph showing the configuration of one of the large tubular air sacs from the lung periphery. At (*a*) is indicated the communication of a terminal bronchiole with the air sac. × 11.22.

Fig. 7. A photograph of a porpoise's lung (*Tursiops truncatus*) stained for elastic tissue for comparison with a similar preparation made from the lung of a manatee. × 30.6.

Plate I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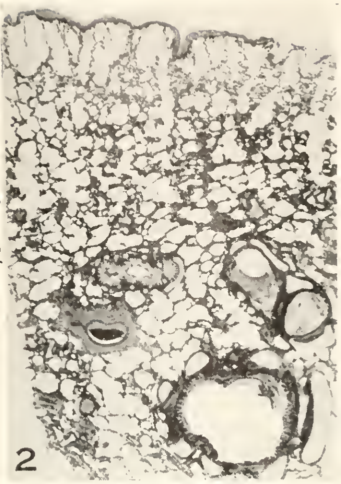
p.v.
br.1
p.a.
br.2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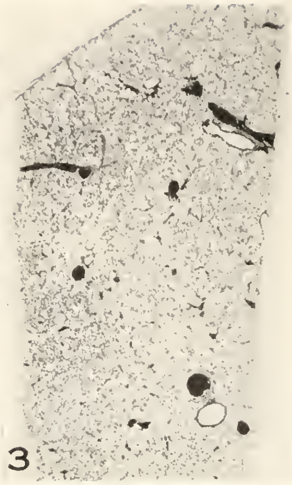
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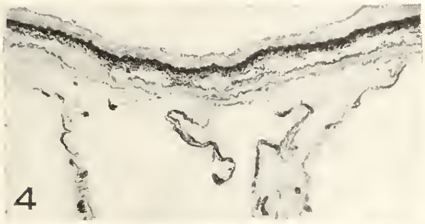
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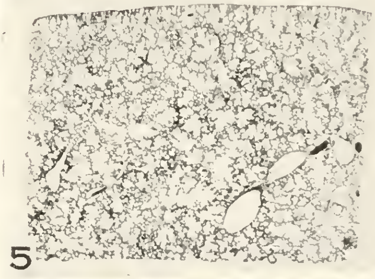
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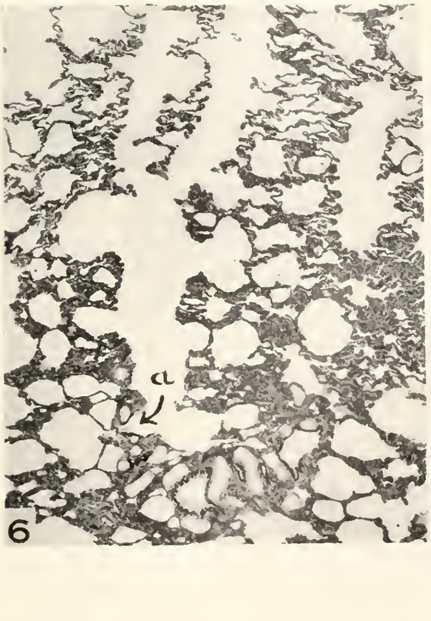
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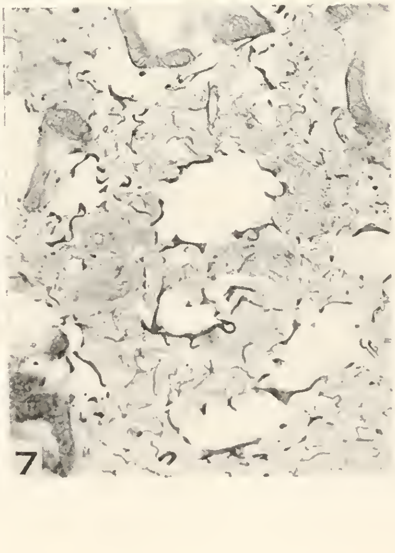
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On taking a block representing about half the diameter of the lung out of the slice of tissue shown in Fig. 1 and preparing sections from it, the finer topography of the manatee lung is revealed. Figure 2 represents about two square centimeters of lung tissue shown at low magnification. For comparison with more familiar lung tissue, sections of normal human lung (Fig. 3) and cat lung (Fig. 5) are shown beside it. The tremendous size of the air sacs and the relatively coarse texture of the lung tissue are at once apparent as compared with either man or cat. The air sacs are many times the size of any terrestrial mammal, and the word "giant alveoli" has been applied by Pick to the similarly large ones encountered in *Halicore dugong*. A further characteristic of both the manatee and dugong is the presence of extremely large air sacs at the border of the lung immediately underneath the pleura. These peripheral air sacs, which are clearly shown in Fig. 2, are roughly cylindrical or tubular in shape contrasted with the smaller polygonal ones in the interior which do not reach the surface. The tubular peripheral air sacs may reach a length of six millimeters and a diameter of from one to two millimeters, whereas the largest ones in the interior are never over three millimeters in greatest length. The tubular air sacs are confined solely to the dorsum of the characteristically flattened lung. The alveolar sacs underlying the pleura on the ventral surface are not elongated. This difference of the two surfaces is related perhaps to the distribution of the bronchial tree which is located on the ventral side of the lung, and sends off its short, stout bronchioles towards the dorsum of the lung.

The terminal bronchioles are short and communicate by wide openings with the large alveolar sacs (Figs. 9 and 11). The openings of the terminal bronchioles into the air sacs are pores having a diameter of from 0.6 to 0.3 mm., hence exceedingly large in diameter compared to similar openings in other mammalian lungs. In the porpoise (*Tursiops*) by comparison the bronchioles are much longer and more slender than in the manatee, and the terminal openings are correspondingly smaller (0.1 to 0.2 mm.). The columnar epithelium which lines the bronchial passages terminates abruptly at the site of union of the bronchioles and the air sacs (Figs. 9 and 11), indicating that the division between the essential respiratory tissue and the conducting passages is located here. The cartilaginous armature of the bronchial tree is highly developed, extending into the walls of the smallest bronchioles. Bits of cartilage can be seen in Figs. 9 and 11 surrounding the smallest bronchioles and their openings into the air sacs. The presence of extensive cartilaginous armature in the bronchial tree is characteristic of all aquatic mammals thus far studied, including the dugong described by Pick. A good description of the arrangement of the cartilage in the trachea and bronchi is given by Pick for the dugong, and, since from my examination of the manatee conditions appear to be the same, I shall give no detailed account of it. Mention should be made of the fact, however, that Pick found calcification present in the larger cartilages of trachea and bronchi. This is not the case in my specimen of the manatee. The individual bits of cartilage in the relatively short, stout terminal bronchioles of the manatee are coarser and more widely separated than in the porpoise (*Tursiops*).

The bronchioles exhibit in their walls a moderate amount of smooth muscle and elastic tissue, nothing like, however, the tremendous amounts of these tissues observed in porpoises by Fiebiger, Lacoste and Baudrimont and myself, where there is a specialized system of myo-elastic sphincters in the terminal bronchioles. Such sphincters are totally lacking in the manatee, as presumably they are also in the dugong, since Pick makes no mention of them. This difference does not discriminate essentially between the sirenians and porpoises, however, for in one of the latter, the small harbour porpoise (*Phocaena*), Lacoste and Baudrimont have not found an elaborated system of sphincters. The elastic tissue in the walls of the bronchioles of the manatee appears predominantly in the form of a lamina of fibers lying between the cartilages and the lumen of the bronchiole. An outer elastic membrane surrounding the cartilages or external to them is not well defined as in *Tursiops*. I have also found in the walls of the bronchioles of the manatee rather extensive accumulations of lymphoid tissue exhibiting here and there "germinal" centers.

The alveolar sacs of the manatee lung, as stated above, are voluminous. They are bounded by excessively heavy septa which, in so far as I can analyze them, appear to be composed of a considerable amount of collagenous fibers, some smooth muscle, and relatively little elastic tissue (Figs. 6, 8, and 10).

The smooth muscle and elastic tissue occurring in the walls of the alveolar sacs of the manatee are not diffusely distributed but bear a definite relationship to the contours of the sacs. It may be seen in any low power field of the manatee lung that the alveolar sacs (both the elongated ones on the periphery, as well as the irregularly shaped ones buried beneath the surface) are subdivided into a series of compartments or lesser sacculations by septal folds (Figs. 6 and 8). These folds have, naturally, free borders which project into the lumen of the alveolar sacs. Moreover, in the tubular air sacs at the surface of the lung, it can be observed that the folds tend to encircle the elongated sacs. Consequently when a sac is cut longitudinally the free edges of the septal folds appear as a series of small knobs as shown in Fig. 8 (*mus.*). These enlarged edges are localities where smooth muscle and elastic tissue intermingled occur in the form of dense bundles (Figs. 8 (*mus.*) and 10), capable, one would presume, of acting as a series of sphincters which control the size of the air sacs. Figure 10 shows these septa stained with resorcin fuchsin to demonstrate elastic tissue. Figure 7 shows for comparison the lung of a porpoise (*Tursiops*) similarly stained. Characteristic of the latter is the smaller size of the alveolar sacs and the much greater abundance of elastic tissue, which is much more diffusely scattered, although here, too, there is a tendency for the elastic tissue to attain its maximum density along the septal margins.

As in the porpoises which have been examined, the pulmonary capillaries form a double bed in the alveolar walls. Each surface of a septum lying between two adjacent air sacs is supplied by separate capillaries separated from one another by collagenous tissue lying in the center of the septum. This double layer of capillaries, not mentioned in Pick's description of the dugong, was probably overlooked in his material. It appears to be a constant arrangement in the obligate aquatic mammals thus far described (*Delphinus*, *Tursiops*, and *Phocaena*).

No lining epithelium can be made out with certainty in the walls of the air-containing, distended alveolar sacs, but in certain areas of

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Explanation of Plate II

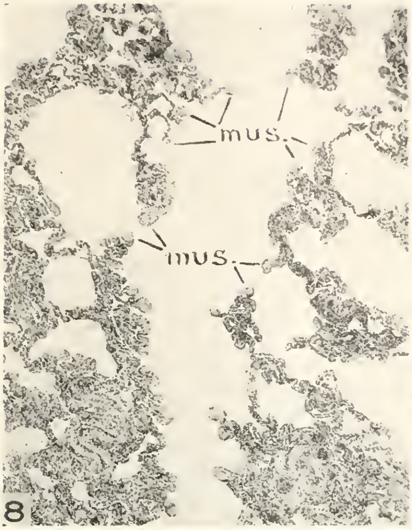
Fig. 8. A photograph of an air sac of the manatee showing the partial subdivision or sacculation of the alveolar sac by stout septa. The free borders of the septa, sometimes slightly club-shaped, are composed of stout bundles of smooth muscle (*mus.*). × 27⅓.

Fig. 9. A photograph showing large air sacs in the interior of the manatee lung showing a communication at one place with a terminal bronchiole (*t. br.*). Numerous bits of cartilage are visible in the walls of the air sacs in this region. × 17.

Fig. 10. A section of manatee lung stained for elastic tissue for comparison with Fig. 8 (both at the same magnification) from which may be gained that the elastic tissue (*el. t.*), similar to the smooth muscle, is condensed at the free borders of the septa which partially subdivide the air sac, the two together forming stout myoelastic bundles. Further, from comparison of this figure with Fig. 7, taken from a porpoise's lung, both stained for elastic tissue and equally magnified, it may be observed how much more abundant elastic tissue is in the lung of the porpoise than in the manatee. × 27⅓.

Fig. 11. A photograph at the point of communication of a terminal bronchiole with an air sac. On the left and bottom of the picture low columnar or cuboidal epithelium of the terminal bronchiole can be seen. Three bits of cartilaginous armature are visible. × 85.

Plate II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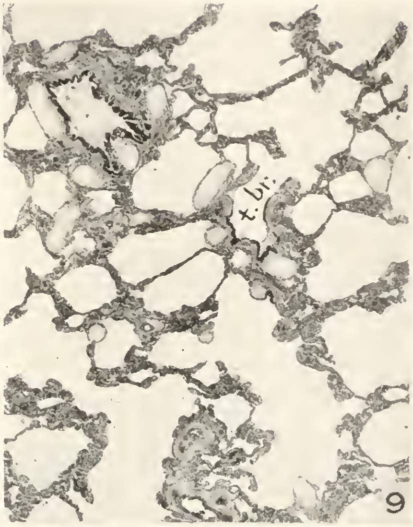
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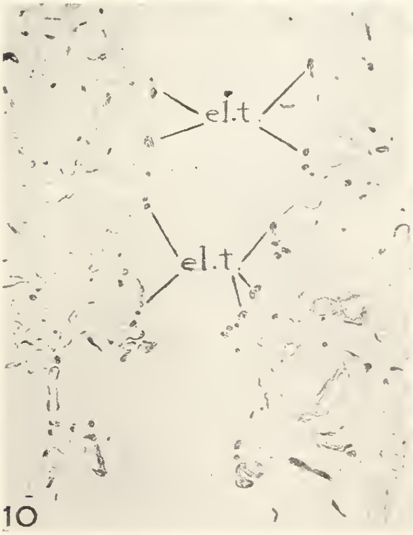
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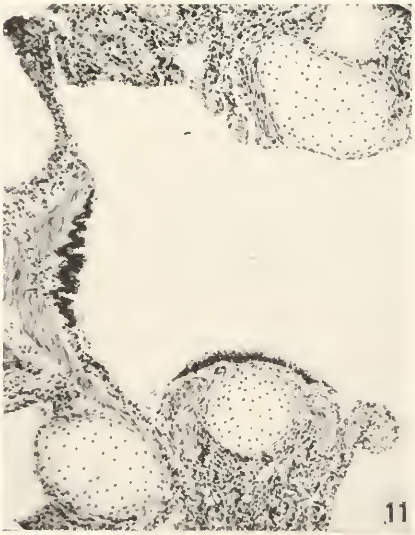
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the tissue, in which the air sacs are atelectatic, a distinct layer of haematoxylin-stained, oval cells, which can be best accounted for as respiratory epithelium, can be observed on the surface of the wall of the alveolus. Similarly the apices of the tubular air sacs beneath the pleura appear in some localities to have undergone partial collapse, giving to the portion of the air sac contiguous to the pleura an almost glandular appearance ascribable to the fact that here also by virtue of the relaxation of the walls a lining of what appears to be epithelial cells has been rendered visible.

The pleura of the manatee is 0.3 to 0.5 mm. thick (Fig. 4). This is thinner than in the porpoise, where it is one-half to one millimeter thick, but on the other hand is materially greater than in terrestrial animals (man, cat, Figs. 3 and 5). Microscopically the pleura is made up of dense bundles of collagenous and elastic fibers besides smooth muscle. It is well vascularized by numerous small arterioles and veins, and contains in addition occasional recognizable lymph vessels. From without in, it is composed of several layers. Outermost, clothed by mesothelial cells, is a layer of collagenous tissue, followed by a heavy lamina of elastic tissue (Fig. 4). Thereupon follows a vascular layer composed of small arteries and veins imbedded in fibrous tissue consisting mostly of white fibers and some smooth muscle bundles. Innermost is a zone of more delicate fibro-elastic tissue containing capillaries.

Discussion and Conclusions

For convenience and brevity in comparing the architecture of the lungs of aquatic mammals thus far known, I have arranged the principal observations in tabular form (Table I). The porpoises and Sirenia have in respect to the histology of the lungs the following characters in common: a very heavy well-vascularized pleura, large alveolar sacs bounded by excessively heavy alveolar walls composed of fibro-elastic and smooth muscle tissue; double layers of capillaries in the alveolar walls, so that single capillaries do not subserve simultaneously the gaseous exchange in two adjacent alveoli; the presence of a well-developed cartilaginous armature extending to the terminations of the smallest bronchioles. For the present these characters may be regarded as the common features of the lungs of mammals which have accommodated themselves to an obligate aquatic existence.

The lungs of the manatee and dugong appear in most respects to be almost identical in structure. They differ specifically from the porpoises in the possession of giant air sacs, with especially large ones lying on the periphery underneath the dorsal pleura. They differ,

TABLE I

	Cetacea			Sirenia	
	Delphinus Fiebiger; Lacoste and Baudrimont	*Tursiops* Wislocki	*Phocaena* Lacoste and Baudrimont	*Halicore* Pick	*Trichechus* Wislocki
Pleura	Thick 0.5 mm. (F)	Thick 0.5–1 mm.	Thick	Thin	Thick 0.3–0.5 mm.
Alveoli					
Size	0.8–1 × 0.5 mm. (F)	1–1½ mm.	Not mentioned	Giant alveoli. 6–10 × 1–3 mm.	Peripheral 4 × 6 × 1–2 mm. Central 2–3 × 2–3 mm.
Septa	Heavy	Heavy	Heavy	Heavy	Heavy
Elastic tissue	Moderate	Rich, forming bundles and plexuses	Moderate	Heavy	Relatively little compared to *Tursiops*, condensed on free borders of alveolar septa
Muscle	Little	Little	Little	Heavy bundles	Heavy bundles on free margins of alveolar septa
Capillaries	Double bed	Double bed	Double bed	Not mentioned	Double bed

TABLE I—*Continued*

	Cetacea			Sirenia	
	Delphinus Fiebiger; Lacoste and Baudrimont	*Tursiops* Wislocki	*Phocaena* Lacoste and Baudrimont	*Halicore* Pick	*Trichechus* Wislocki
Bronchioles					
Size	No specific details	Slender, long, much branched. Terminal openings 0.1 to 0.2 mm.	No specific details	No specific details	Voluminous, short. Terminal openings 0.3 to 0.6 mm.
Cartilage	Into finest bronchioles	Into finest br. Abundant, small, almost continuous links	Into finest bronchioles	Into finest bronchioles	Into finest bronchioles. Fewer, larger, more discontinuous bits than in *Tursiops*
Elastic tissue	Heavy throughout	Heavy throughout	Heavy throughout	Abundant	Relatively little compared to *Tursiops*
Muscle	System of heavy muscle sphincters	System of heavy muscle sphincters	Scant. No system of sphincters	Well developed, but no sphincters	Moderate, but no system of sphincters
Epithelium	Flat in smallest bronchioles which have respiratory function	Flat in smallest bronchioles which have respiratory function	Cuboidal, without cilia or mucous cells	Flat in smallest bronchioles	Cuboidal or columnar throughout with no respiratory function. Mucous secretion abundant
Blood vessels	Helicine arterioles (C and B)	No special plexuses	Plexiform veins between groups of alveoli	No mention	No special plexuses

moreover, from the porpoises in not having a system of specialized myo-elastic valves in the smallest bronchioles, as well as by virtue of the fact that the terminal bronchioles are short, wide in diameter, and lined by cuboidal or columnar epithelium.

The lungs of the porpoises themselves, in view of the recent study of *Phocaena* by Lacoste and Baudrimont, are apparently not all equally specialized. Characteristic of them as a group is the possession of a dense pleura and rather large air sacs with stout walls containing much elastic tissue and a double layer of capillaries. The complicated series of muscular valves present in the smallest bronchioles of *Delphinus* and *Tursiops* is not present, however, in *Phocaena*, the small harbour porpoise.

In conclusion the author does not wish to speculate upon the functional implications of the anatomical structures described. This has been done briefly before for the porpoise by Fiebiger (1916) and Wislocki (1929) and for the whales in general in an extensive although entirely speculative manner by Lacoste and Baudrimont (1933). Reference should also be made to the interesting speculations of Irving (1934) on the ability of warm-blooded animals to survive without breathing.

The present study brings out the close similarity in lung structure in the two families of Sirenia. It demonstrates likewise the marked dissimilarities between the sirenian lungs and those of the porpoises belonging to the order of whales. Although there are certain distinctive features common to the lungs of the aquatic mammals thus far investigated, the pronounced differences found to exist indicate that various avenues of specialization have been followed in adapting to aquatic modes of life. The capacity of various aquatic animals to remain submerged and the depths to which they may dive doubtless vary considerably and the degree to which they possess these abilities is probably correlated with differences in lung structure. The Sirenia presumably submerge to lesser depths and are not able to remain under so long as the majority of whales (Parker, 1922, 1932). In this connection it will be of interest to learn eventually how closely the lungs of the large whales resemble those of the porpoises. The seemingly less specialized lungs of the small harbour porpoise (as described by Lacoste and Baudrimont) may be related to the possibility that this small species, in contrast to the larger dolphins (*Delphinus* and *Tursiops*), is not able to submerge for long periods or to attain any great depths. For the present the complicated system of bronchiolar valves possessed only by the two larger species appears to be an ideal specialization to meet the requirements of prolonged submerging and the attainment of considerable depth.

LITERATURE CITED

Fiebiger, J., 1916. Über Eigentümlichkeiten im Aufbau der Delphinlunge und ihre physiologische Bedeutung. *Anat. Anz.*, **48**: 540.

Irving, L., 1934. On the Ability of Warm-blooded Animals to Survive without Breathing. *Scient. Month.*, **38**: 422.

Lacoste, A., and A. Baudrimont, 1926. Sur quelques particularités histologiques du poumon du Dauphin, et leur adaptation fonctionelle à la plongée. *Bull. de la Soc. Scient. d'Arachon*, T. **3**.

Lacoste, A., and A. Baudrimont, 1933. Dispositifs d'adaptation fonctionelle à la plongée dans l'appareil respiratoire du Marsouin (Phocaena communis, Less.). *Arch. d'anat., d'histol. et d'embryol.*, **17**: 1.

Parker, G. H., 1922. The Breathing of the Florida Manatee (Trichechus latirostris). *Jour. Mamm.*, **3**: 127.

Parker, G. H., 1932. The Respiratory Rate of the Common Porpoise. *Jour. Mamm.*, **13**: 68.

Pick, F. K., 1907. Zur feineren Anatomie der Lunge von Halicore dugong. *Arch. f. Naturgesch.*, **73**: 245.

Wislocki, G. B., 1929. On the Structure of the Lungs of the Porpoise (Tursiops truncatus). *Am. Jour. Anat.*, **44**: 47.

THE DEVELOPMENTAL STAGES OF LABIDOCERA

MARTIN W. JOHNSON

(*From the Scripps Institution of Oceanography of the University of California, La Jolla, California*)

INTRODUCTION

Within the past year there has been an increased effort directed towards the study of the development and life histories of marine copepoda. Five publications have recently appeared (Nicholls, 1934; Gurney, 1934; Campbell, 1934; Johnson, 1934*a*, 1934*b*) describing in detail the development of a total of seven marine calanoids. Prior to these works only nine species of this group had been investigated as to their complete development. Gurney (1934) has attributed this lag in investigation of the development of this, the most important of the marine plankton copepod groups, to the difficulty experienced in laboriously tracing out the life histories from the various stages as collected from the plankton, a difficulty further enhanced by the failure of many species to carry eggs. More success has been obtained in the study of fresh water calanoids, due to the greater ease with which they may be cultured in the laboratory. Thus far only one marine calanoid (*Calanus finmarchicus*) has been successfully reared through all the stages to the adult (Lebour, 1916). Gibbons (1933) has cultured this species through the nauplius stages. *Euchaeta norvegica* has been reared through the nauplius stages into the first copepodid; the nauplii apparently being sustained by a large store of yolk (Nicholls, 1934).

The species included in the present study are *Labidocera trispinosa* and *Labidocera jollæ*, which were described by Esterly (1905, 1906) from plankton collections made off the southern California coast. The former is reported as fourth in abundance of the copepods occurring in this region, and is at times very numerous above one hundred fathoms (Esterly, 1912). It is evidently more or less sporadic in occurrence and is found only sparsely in inshore collections. *Labidocera jollæ* is relatively more scarce, judging from the infrequent reference to it in Dr. Esterly's works. My inshore collections indicate that its nauplius stages are as a rule less numerous than those of *Labidocera trispinosa* and that the two species reproduce concurrently during the summer and autumn, and to a lesser extent well into winter. The spring season has not been investigated.

These are the only two species of *Labidocera* reported from this region. The nearest relative whose nauplius larvae might on superficial examination be confused with those of the present species is *Epilabidocera amphitrites*. This species, however, has not been found south of San Francisco Bay and its larval stages are known (Johnson, 1934*b*) and can on careful examination be quite readily distinguished from the present species.

In a preliminary investigation of the seasonal occurrence of zoöplankton at La Jolla, Murphy (1924) concluded that 70–80 per cent of the total plankton was made up of immature forms whose generic identity is not known. Copepod nauplii formed the major part of this percentage, but to what extent the present species of *Labidocera* entered into the computations is not known. Thus far I have found them to play only a minor rôle in local inshore waters.

PROCEDURE

The material used in the present study was obtained from surface plankton collections taken at the Scripps Institution pier. *Labidocera jollæ* female copepodid V was, however, found only in an offshore collection put at my disposal through the courtesy of Dr. E. G. Moberg. This collection also provided material for checking the adult stages of both species. Both living and preserved specimens were employed in working out the successive stages. The identity of the larvæ was established by experimentally rearing the last nauplius stage through the critical moult to the first copepodid stage and linking this with the successive copepodid stages to the adult, which proved to be of the genus *Labidocera*.

The nauplius larvae of the two local species of Labidocera are separable on small but consistent specific characters mentioned later. A total of six specimens of the last nauplius stage of *Labidocera trispinosa* were reared through the critical moult and the resulting first copepodid examined by complete dissection. Four specimens of *Labidocera jollæ* were thus reared and examined, and many specimens of both species were examined from plankton collections. It is interesting to note that the specific differences appear no greater in the first copepodid stage than in the preceding nauplius stage, but in the following instars the species are easily separated by the clearly visible cephalic hooks present in *Labidocera jollæ* but wanting in *Labidocera trispinosa*, and by the longer first antennae of the latter species.

All drawings were made with the aid of a camera lucida.

LABIDOCERA TRISPINOSA

The Nauplius Stages

The early development is divided into the characteristic six nauplius stages. Pronounced specific differentiation does not appear until the second stage, and each following moult accentuates or brings to light new differentiations.

Contrasted with nearly all copepod nauplii, the larvae of *Labidocera trispinosa* and *Labidocera jollæ* are noticeably elongated. This elongation is, however, only of an intermediate nature when compared with the elongation of the later larvæ of such forms as *Rhincalanus* (Gurney, 1934, Figs. 3, 4, 5) and a local larva tentatively classified as *Pontellopsis occidentalis*. The appendages of *Labidocera trispinosa* are also relatively long and the first antennæ are normally directed straight forward and in contact nearly their whole length. In the later nauplius stages the distal segment of the first antenna is faintly orange in color; the color increasing in intensity toward the tip. Similar pigment is also present in the second antenna, but here the greatest intensity is in the first basipod. The mandibles are nearly colorless. A little of the pigment is visible on the ventral surface of the body and in the alimentary canal where a small splash of red also appears in some specimens. The eye is very dark reddish-brown. The general pigmentation is quite variable in intensity and specimens that have been kept alive in the laboratory a day or longer may become quite colorless.

The labrum is long and rather wide and only very sparsely armed with short weak setæ.

The plumose nature of the appendage setæ can in some instances be seen only with difficulty and is here indicated only in the figures for the sixth nauplius stage.

Nauplius Stage I (*Plate I, Fig. 1*)

Body.—0.13–0.14 mm. long, oval, posterior end bearing two small spines.

First antenna (Plate IV, Fig. 5).—Three segments, the first short and with one short ventral seta; the second somewhat longer and bearing ventrally one very short and one long seta; the third or distal segment bearing terminally three long setae and ventrally near the tip a short spine.

Second antenna.—First basipod with one small masticatory hook. Second basipod with one small masticatory hook and an adjacent small outwardly directed spine. Endopod of one segment with two terminal setæ and one lateral seta. Exopod of six segments, the first two fused, the first with no seta, 2–5 with one seta each and the sixth with two setæ.

Mandible.—First basipod with a small rounded chewing process bearing a small spine. Second basipod with two inner spines. Endopod of two completely fused segments indicated only by the presence of three short weak spines on the first and one very short and two long setae on the second. Exopod of four segments, 1–3 with one seta each, the fourth with two setæ.

Nauplius Stage II (*Plate I, Fig. 2*)

Body.—0.175–0.201 mm. long (average of 11 measurements, 0.185 mm.) terminating posteriorly in one long heavy setose spine and one shorter dorsally directed plumose seta at its right. Partially surrounding the base of each of these is a number of very short fine spines.

First antenna (Plate IV, Fig. 6).—The first and second segments as in I; the distal segment bears at the tip three long plumose setæ and one shorter, lighter accessory seta and the dorsal and ventral margins each bear a separate row of fine hair-like setæ.

Second antenna.—The first basipod with one masticatory hook. Second basipod with one long heavy masticatory hook, an adjacent small outwardly directed spine and one small spine situated distally near the endopod. Endopod with three long terminal setæ and one long lateral seta bearing a small smooth process near its proximal end. Exopod as in I but with two setæ on the second segment.

Mandible.—First and second basipods as in I. Endopod as in I but with increased strength of armature. Exopod as in I but with two setæ on the first segment indicating a segmentation which remains latent until the first copepodid stage is reached.

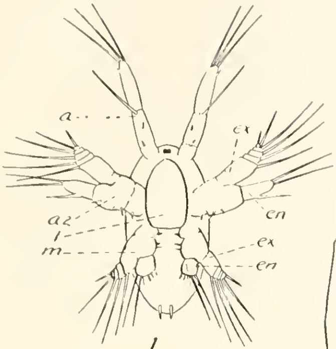
Explanation of Plate I

Labidocera trispinosa

Fig. 1. Nauplius Stage I.
Fig. 2. Nauplius Stage II.
Fig. 3. Nauplius Stage III.
Fig. 4. Nauplius Stage IV.
Fig. 5. Nauplius Stage V.

Abbreviations: *a*—first antenna.
a2—second antenna.
cp—chewing process.
en—endopod.
ex—exopod.
l—labrum.
lh—lateral hook.
m—mandible.
mx—first maxilla.
vh—ventral hook.

Plate I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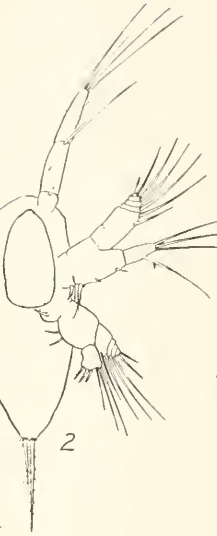

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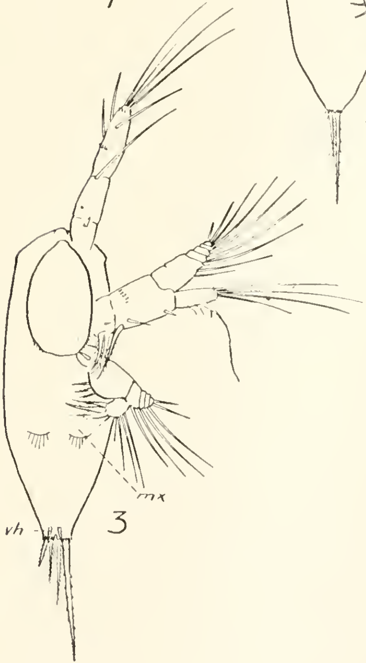

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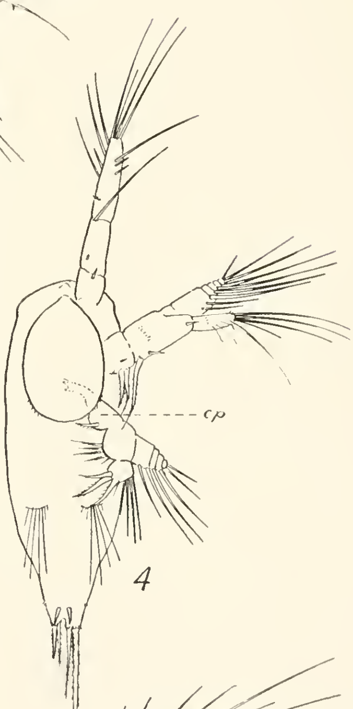

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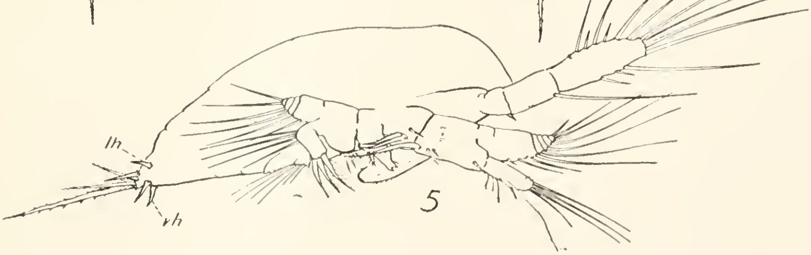

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Nauplius Stage III (Plate I, Fig. 3)

Body.—0.215–0.271 mm. in length (average of 23 measurements, 0.235 mm.). Posterior end armed with one long left and one short right terminal setose spine, and one flexible inner plumose seta in conjunction with each spine. Just anterior to the terminal armature there is now one pair of ventral hooks.

First Antenna (Plate IV, Fig. 7).—Segments one and two as in II, the distal segment with four terminal setæ, two long dorsal marginal setæ, one long ventral marginal seta, and a transverse series of minute spines on the inner margin near the middle. The fine hair-like setæ found on the distal segment in II are now wanting.

Second Antenna.—First basipod with two long strong masticatory hooks upon a common base and adjacent to these one small spine. Second basipod as in II but with also a transverse series of minute spines situated on the ventral proximal portion of the segment. Endopod as in II but with four terminal setæ and two additional fine hair-like setæ near the origin of the single long lateral seta. Exopod as in II but with three setæ each on the second and terminal segments.

Mandible.—First basipod as in II. Second basipod with three inner spines. Endopod with three strong hook-like spines and one very weak basal seta on the first segment and four slender setæ on the second segment. Exopod as in II.

First Maxilla.—Bud fringed with short hairs, discernible only with difficulty in some specimens.

Nauplius Stage IV (Plate I, Fig. 4)

Body.—0.250–0.325 mm. long (average of 14 measurements 0.278 mm.). The caudal armature is like that in Stage III with the addition of a series of minute lateral spines marking the location where the lateral hooks are destined to appear in the following stage.

First Antenna (Plate IV, Fig. 8).—First and second segments unchanged; distal segment with four terminal setæ, one short and three long dorsal marginal setæ, one long and two short ventral marginal setæ and a transverse series of minute spines on the inner side.

Second Antenna.—First and second basipods as in III. Endopod as in III but with also a few very minute spines grouped on the lateral surface. Exopod as in III.

Mandible.—First basipod with the chewing process much enlarged and terminating in two teeth and a small lateral seta. Proximally the process bears a heavy setose spine. Second basipod with five inner spines. Endopod and exopod as in III.

First Maxilla.—Bud with slender weakly chitinized setæ.

Second Maxilla.—Poorly defined bud. (Visible only in some specimens nearing ecdysis.)

Nauplius Stage V (Plate I, Fig. 5)

Body.—0.310–0.370 mm. in length (average of 14 measurements, 0.332 mm.). Posterior end armed as in IV but including also one pair of lateral hooks similar to the ventral pair.

First Antenna (Plate IV, Fig. 9).—Unchanged except for increase to four long and two short (alternating with the long) dorsal marginal setæ and one long and two short ventral marginal setæ.

Second Antenna.—First basipod unchanged. Second basipod unchanged but for addition of one very small spine adjacent to the masticatory hook, thus making two small spines in this location. Endopod as in IV but with three fine hair-like setæ near the base of the single long lateral seta. Exopod as in IV but with four setæ on the second segment.

Mandible.—As in IV.

First and Second Maxilla.—Rudimentary.

Maxilliped.—Bud.

Nauplius Stage VI (Plate II, Fig. 1)

Body.—0.360–0.420 mm. long (average of 11 measurements, 0.386 mm.). Caudal armature as in Stage V. Just anterior to the lateral hooks there is on each side a series of very minute lateral spines. The rudimentary legs which in some specimens of Stage V can be seen as blocks of undifferentiated tissue are now quite distinct, but the first maxillæ are still poorly defined.

First Antenna (Plate IV, Fig. 10).—As in Stage V but with a total of five ventral marginal setæ on the distal segment.

Second Antenna.—First and second basipods as in V. Endopod as in V but with a total of five terminal setæ. Exopod as in V.

Mandible.—As in Stage V but with increased strengthening of the masticatory portions and with a total of six setæ on the inner margin of the second basipod.

First and Second Maxilla.—Rudimentary.

Maxilliped.—Bud.

First and Second Legs.—Rudimentary.

The Copepodid Stages

Following the critical moult there is a succession of six copepodid stages, the last of which is the adult animal. Living animals display a rather faint dark orange coloration of the first and second basipods of the second antennæ, and some of this pigment is also evident in the posterior end of the body. A few dark spots occur along the mid body and a faint green is noted along the alimentary canal. The color intensity varies greatly and is usually more pronounced in the older specimens.

From the first copepodid stage, the animals swim in the fashion apparently typical of the *Pontellidæ*, i.e. with quick sweeps of the second antennæ and with rhythmic dorsal ventral motion of the urosome. Thus, when swimming leisurely, the animal gives the appearance of a hovering bird.

Copepodid Stage I (*Plate II. Fig. 2*)

Length 0.498–0.590 mm. (average of 5 measurements 0.543 mm.) to the end of caudal rami. Thorax [1] of four segments, abdomen of one segment. The abdomen and caudal rami are symmetrical and each ramus bears four long terminal setæ, one short lateral seta (which in the later stages assumes the character of the regular terminal setæ), and one dorsal seta. Dorsal cuticular lenses are poorly defined and partially surrounded by pigment bodies. The rostral processes which are typical of the later stages are wanting, the rostrum being only a rounded blunt protuberance.

First Antenna.—Nine evident segments.

Second Antenna.—First basipod with one seta. Second basipod with two setæ. Endopod of two segments, the first fused with the second basipod, and bearing two outer setæ, the second segment forming a lateral and a terminal lobe each bearing a distinct group of setæ indicating the fusion of two segments homologous to the two clearly defined end segments of the endopod of the mandible. The lateral lobe bears a group of three setæ, and the terminal lobe, six setæ. The exopod consists of five evident segments, the first short and bearing one inner seta, the second long and bearing three setæ. The remaining end segments are short and very obscure and bear a total of eight setæ.

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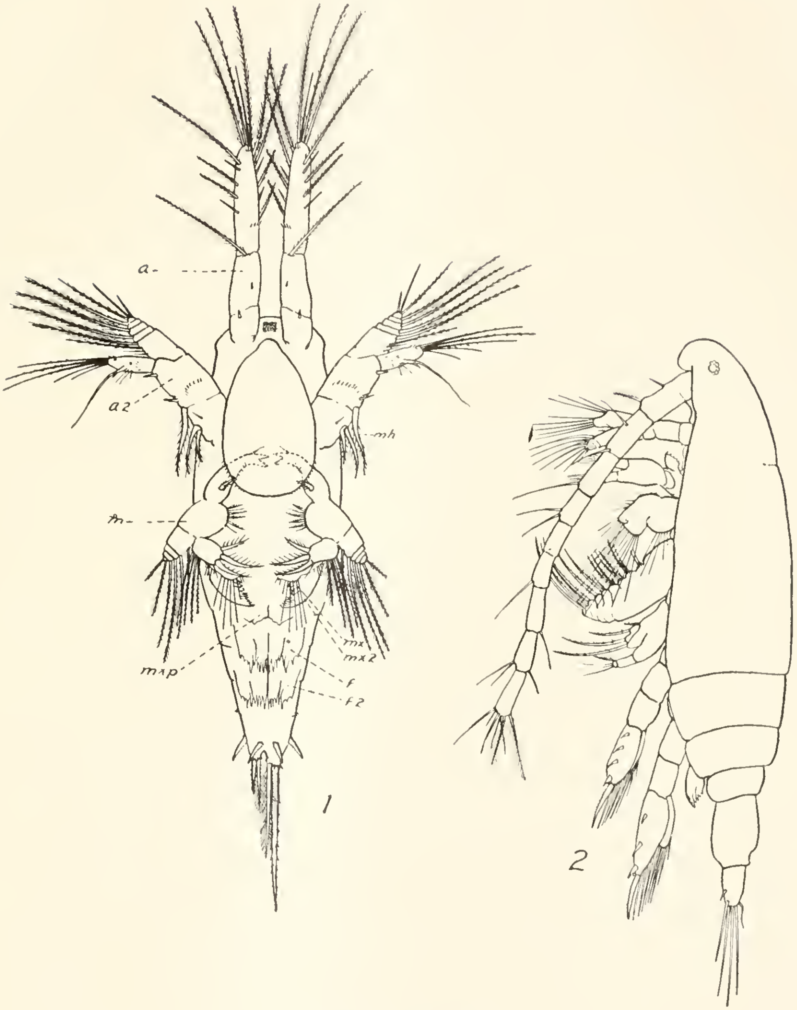
[1] The term 'thorax' used in connection with these species designates that portion of the body bearing visible feet plus the next segment to the posterior. Thus the adult animal is considered to possess a total of six thoracic segments of which the genital segment is the last to appear.

Plate II



Labidocera trispinosa

Fig. 1. Nauplius Stage VI.
Fig. 2. Copepodid Stage I, lateral.

Abbreviations: *a*—first antenna.
*a*2—second antenna.
f—first legs.
*f*2—second legs.
m—mandible.
mh—masticatory hook of second basipod.
mx—first maxilla.
*mx*2—second maxilla.
mxp—maxilliped.

Mandible.—Mandibular blade with four teeth, a small spine, and several fine short setæ. Palp with a very short first basipod; a long second basipod with four slender setæ; an endopod of two short segments, the first with a distal group of four long setæ, and the second with a terminal group of six long setæ; an exopod of five segments, 1–4 with one long seta each and the fifth with two shorter slender setæ.

First Maxilla.—The structure of this appendage is like that of the adult except for less obvious segmentation and smaller number of setæ on certain lobes. Gnathobase or first inner lobe with eleven short spines; second inner lobe with three long strong setæ; epipod or outer setiferous plate bears four long slender setæ; the third inner lobe situated on a very short segment bears three long setæ, while the outer margin of this segment bears a single long seta. The following segment bears three slender inner setæ and is fused with the first segment of the exopod which bears three lateral setæ. The second or terminal segment of the exopod bears five long coarse setæ. Endopod of two poorly defined segments, the terminal one with seven long coarse setæ.

This appendage is identical with that of *Labidocera jollæ* which is figured for the adult in Plate IV, Fig. 13.

Second Maxilla.—Uniramose and strongly built. First basipod with two endites each bearing one short and one long seta and a number of fine spines at their bases. Second basipod with two endites each with one short and two long setæ; the first endite also bears a number of very fine spines. Endopod of five poorly defined segments, the first segment slightly the longest and bearing one long and one short seta, segments 2–4 each with one long seta, and the fifth segment with one long and one short seta. All the setæ of the appendage are strong and coarsely setose.

Maxilliped.—Uniramose. The first basipod enlarged and consisting of three lobes, the first with one short setose seta, the second with one long and one short setose seta, the third with one long setose seta. The second basipod is narrow and bears no setæ. Endopod of two segments, the first with one distal plumose seta, the second with three terminal smooth setæ.

First Leg.—First and second basipod with no setæ. Endopod of one segment with seven setæ. Exopod of one segment with four outer spines, a terminal blade and three setæ.

Second Leg.—First and second basipods with no setæ. Endopod of one segment with six setæ. Exopod of one segment with three outer spines, a terminal blade and three setæ.

Third Leg.—Rudimentary.

Copepodid Stage II

Length 0.720–0.797 mm. (average of 5 measurements, 0.767 mm.). Thorax of five segments, abdomen of one segment. Dorsal cuticular lenses and rostral processes present. Urosome and caudal rami symmetrical.

First Antenna.—Fourteen segments.

Second Antenna.—As in I but with five setæ on the lateral lobe and six setæ on the terminal lobe of the distal segment of the endopod.

Mandible.—As in I but with five mandibular teeth, and with six setæ on the terminal segment of the endopod.

First Maxilla.—As in I but with six setæ on the epipod and four setæ on the third inner lobe.

Second Maxilla.—As in I.

Maxilliped.—As in I but with two short setæ on the first lobe of the first basipod; and with an endopod of three segments, the first with one long and one short plumose seta, the second with one long plumose seta, and the third with three long smooth setæ.

First Leg.—First basipod with one inner seta; second basipod with no seta. Endopod of one segment with eight setæ. Exopod of two segments, the first with one outer spine, the second with three outer spines, a terminal blade, and four setæ.

Second Leg.— First basipod with one seta; second basipod with no seta. Endopod of one segment with eight setæ. Exopod of two segments, the first with one outer spine, the second with two outer spines, a terminal blade, and four setæ.

Third Leg.—First and second basipods with no setæ. Endopod of one segment with six setæ. Exopod of one segment with three outer spines, a terminal blade, and three setæ.

Fourth Leg.—Rudimentary.

Copepodid Stage III

Length 1.03–1.11 mm. (average of 5 measurements 1.064 mm.). Thorax of six segments, abdomen of one segment. Posterior end of body symmetrical.

First Antenna.—Nineteen segments.

Second Antenna.—As in II.

Mandible.—As in II.

First Maxilla.—As in II but with eight setæ on the epipod and four setæ on the first segment of the endopod.

Second Maxilla.—As in II.

Maxilliped.—As in II but with two short plumose setæ at the base of the one long seta on the third lobe of the first basipod, and with an endopod of five segments. The first segment of the endopod is very short and indistinctly defined and bears two long plumose setæ; the second segment is long and bears one plumose seta; the third and fourth segments are shorter and each bear distally one plumose seta; the fifth segment is very short and terminates in three smooth setæ.

First Leg.—First basipod with one inner seta; second basipod with no seta. Endopod of one segment with nine setæ. Exopod of two segments, the first with one outer spine and one inner seta, the second with three outer spines, a terminal blade and four setæ.

Second Leg.—First basipod with one inner seta; second basipod with no seta. Endopod of one segment with nine setæ. Exopod of two segments, the first with one outer spine and one inner seta, the second with three outer spines, a terminal blade and five setæ.

Third Leg.—First basipod with one inner seta; second basipod with no seta. Endopod of one segment with eight setæ. Exopod of two segments, the first with one outer spine, the second with two outer spines, a terminal blade, and four setæ.

Fourth Leg.—First and second basipods with no setæ. Endopod of one segment with six setæ. Exopod of one segment with three outer spines, a terminal blade and three setæ.

Fifth Leg.—Rudimentary.

Copepodid Stage IV

Length female 1.34–1.49 mm. (average of 8 measurements, 1.40 mm.); male 1.23–1.41 mm. (two specimens). Thorax of six segments, abdomen of two segments. The corners of the fifth thoracic segment are symmetrical and slightly pointed, and the urosome is also symmetrical.

First Antenna.—Twenty-three segments.

Second Antenna.—As in III but with seven setæ on the lateral lobe of the terminal segment of the endopod, and with an additional slender seta on the terminal segment of the exopod.

Mandible.—As in III but with seven setæ on the terminal segment of the endopod.

First Maxilla.—As in III but the epipod and exopod each with nine setæ.

Second Maxilla.—As in III but with the addition of two setæ at the base of the two long setæ on the first endite of the first basipod.

Maxilliped.—As in III but with an additional small seta at the base of the two longer setæ on the second lobe of the first basipod. The second basipod is finely serrate on the anterior surface, and there are now two plumose setæ on the distal end of the second segment of the endopod.

This appendage is now essentially the same as in the adult stage (Plate IV, Fig. 23). The short first segment of the endopod, however, becomes so obscure by fusion to the second segment in the later stages that the endopod appears to have only four segments.

First Leg.—First basipod with one inner seta; second basipod with no seta. Endopod of one segment with nine setæ. Exopod of two segments, the first with one outer spine and one inner seta, the second with three outer spines, a terminal blade, and four setæ.

Second Leg.—First basipod with one inner seta; second basipod with no seta. Endopod of one segment with ten setæ. Exopod of two segments, the first with one outer spine and one inner seta, the second with three outer spines, a terminal blade, and five setæ.

Third Leg.—First basipod with one inner seta; second basipod with no seta. Endopod of one segment with nine setæ. Exopod of two segments, the first with one outer spine and one inner seta, the second with three outer spines, a terminal blade, and five setæ.

Fourth Leg.—First basipod with one inner seta; second basipod with no seta. Endopod of one segment with eight setæ. Exopod of two segments, the first with one outer spine, the second with three outer spines, a terminal blade, and five setæ.

Fifth Legs, Female (Plate IV, Fig. 14).—Biramose, small and symmetrical. The second basipod bears on its posterior surface a short plumose seta. Endopod represented by a short smooth segment. Exopod of one segment with one outer spine near the middle and one near the tip. At the tip there is one terminal spine and adjacent to it on the inner side is a very small spine which in the later stages develops into the larger terminal point.

Fifth Legs, Male (Plate IV, Fig. 19*a*).—Biramose and asymmetrical. The second basipod and the endopod are like the corresponding parts in the female. Right exopod slightly longer than the left and with two segments indicated. Each exopod bears two small outer spines and a terminal point.

In this stage the sexes apparently can be distinguished only by the structure of the fifth legs.

Copepodid Stage V

Length, female 2.04–2.06 mm. (three measurements). Thorax of six segments, abdomen of two segments. The fifth thoracic segment with sharply pointed corners. Posterior portion of body symmetrical and without protuberance on genital segment. No males were found of this species in this stage.

First Antenna.—Twenty-three segments discernible.

Second Antenna.—As in IV.

Mandible.—As in IV with setæ of terminal endopod segment increased to eight.

First Maxilla.—Segmentation more defined and increased strength of armature.

Second Maxilla. As before but with a total of five setæ on the first endite of the first basipod.

Maxilliped.—No change in structure.

First Leg.—First basipod with one inner seta; second basipod with no seta. Endopod of two segments, the first with three inner setæ, the second with six setæ. Exopod of three segments, the first and second with one outer spine and one inner seta, the third with two outer spines, a terminal blade, and four setæ.

Second Leg.—First basipod with one inner seta; second basipod with no seta. Endopod of two segments, the first with three inner setæ, the second with eight seta. Exopod of three segments, the first and second with one outer spine and one inner seta, the third with three outer spines, a terminal blade, and five setæ.

Third Leg.—First basipod with one inner seta; second basipod with no seta. Endopod of two segments, the first with three inner setæ, the second with eight setæ. Exopod of three segments, the first and second with one outer spine and one inner seta, the third with three outer spines, a terminal blade, and five setæ.

Fourth Leg.—First basipod with one inner seta; second basipod with no seta. Endopod of two segments, the first with three inner setæ, the second with seven setæ. Exopod of three segments, the first and second with one outer spine and one inner seta, the third with three outer spines, a terminal blade, and five setæ.

Fifth Leg, Female (Plate IV, Fig. 15).—Similar to Stage IV, with the small inner terminal spine enlarged to a heavy terminal point.

Copepodid Stage VI, Adult

The original description given for the adult stage of this and the following species is not complete. It is therefore desirable to include here also brief descriptions of this stage.

Female (Plate V, Fig. 1).—Length 2.50–2.82 mm. (average of 7 measurements, 2.81 mm.). The measurements given by Esterly (1905, p. 202) for the adult female is 1.6 mm.; only a little greater than is here given for copepodid Stage IV. The structure of the adult appendages and body segmentation is essentially the same as in Stage

V. In Plate IV are figured the fifth legs (Fig. 16), the mandible (Fig. 12), and the maxilliped (Fig. 23). The last two named appendages are the same in both sexes.

Male (Plate V, Fig. 2).—Length 2.14–2.47 mm. (average of 7 measurements, 2.31 mm.). This agrees rather closely with Esterly's maximum figure which is 2.2 mm. The abdomen consists of four segments. The right posterior corner of the fifth thoracic segment is produced into a slender curved spine, and adjacent to it on the posterior margin on the same side there are two to three smaller spines. Esterly (1905, p. 202) notes only two such spines. The fifth legs (Plate IV, Fig. 22) are as in the original description but with a rudimentary endopod on the left foot.

LABIDOCERA JOLLÆ

The adults of *Labidocera jollæ* and *Labidocera trispinosa* are easily distinguished by sharply defined characters, but the nauplius larvæ of the species are very similar and can be distinguished only by small but yet definite differences.

Each developmental stage of *Labidocera jollæ* was also worked out carefully and will be briefly compared with those of *Labidocera trispinosa* in all the essential characters, stressing mainly the points of difference.

Nauplius Stage I was not found or could not be distinguished from the corresponding stage referred to *L. trispinosa*. (The nauplius examined was referred to this species in view of its greater numbers.) A study of the following nauplius stages will show that the separation of the two species in Stage I must depend mainly, if not wholly on the comparative slenderness of the masticatory hooks occurring on the first and second basipod of the second antenna. These hooks are only weakly developed in this stage.

In the following nauplius stages *L. jollæ* is usually slightly the larger of the two species, the first antennæ and the long caudal spine are relatively shorter, and the pigmentation of the body is more pronounced and of a definite blue-green cast. When at rest the first antennæ are directed straight forward.

Nauplius Stage II (*Plate III, Fig. 1*)

Length 0.200–0.211 mm. (two specimens measured). The anatomical feature distinguishing the species in this stage is a very slender masticatory hook on the second basipod of the second antenna as compared with the heavy, well developed corresponding hook in *L. trispinosa* (Plate IV, Figs. 1 and 2, *mh*). That this character is a constant one is supported by its persistence in combination with other specific characters that will be mentioned for the later stages. The short spine appearing on the distal portion of the second basipod of the second antenna of *L. trispinosa* is wanting in *L. jollæ*. Its first appearance is in Stage III. This spine and also the other spines occurring on the second antenna are more slender in the latter species in all stages.

Nauplius Stage III

Length 0.260–0.270 mm. (three measurements). The only additional difference in this stage is the appearance of a transverse series of very minute spines on the second basipod of the second antenna. In *L. jollæ* the series is located on the distal half of the segment while in *L. trispinosa* it is on the proximal half. This difference persists throughout all of the stages but in the older larvæ a few similar small spines appear also on the proximal half of the segment in *L. jollæ*.

Nauplius Stage IV

Length 0.265–0.319 mm. (average of six measurements 0.304 mm.). In this stage the development of the larval chewing process is completed in both species. In *L. jollæ* the process terminates in three teeth and a small spine, while in *L. trispinosa* it terminates in only two teeth and a small spine (Plate I, Fig. 4, *cp;* Plate IV, Figs. 3 and 4). The greater number of mandibular teeth in the former species is reflected in the copepodid stages, in the first of which the number is in the same ratio as in the nauplius stage.

Nauplius Stage V

Length 0.390–0.426 mm. (average of five measurements 0.398 mm.). No added specific changes.

Nauplius Stage VI (Plate III, Fig. 2)

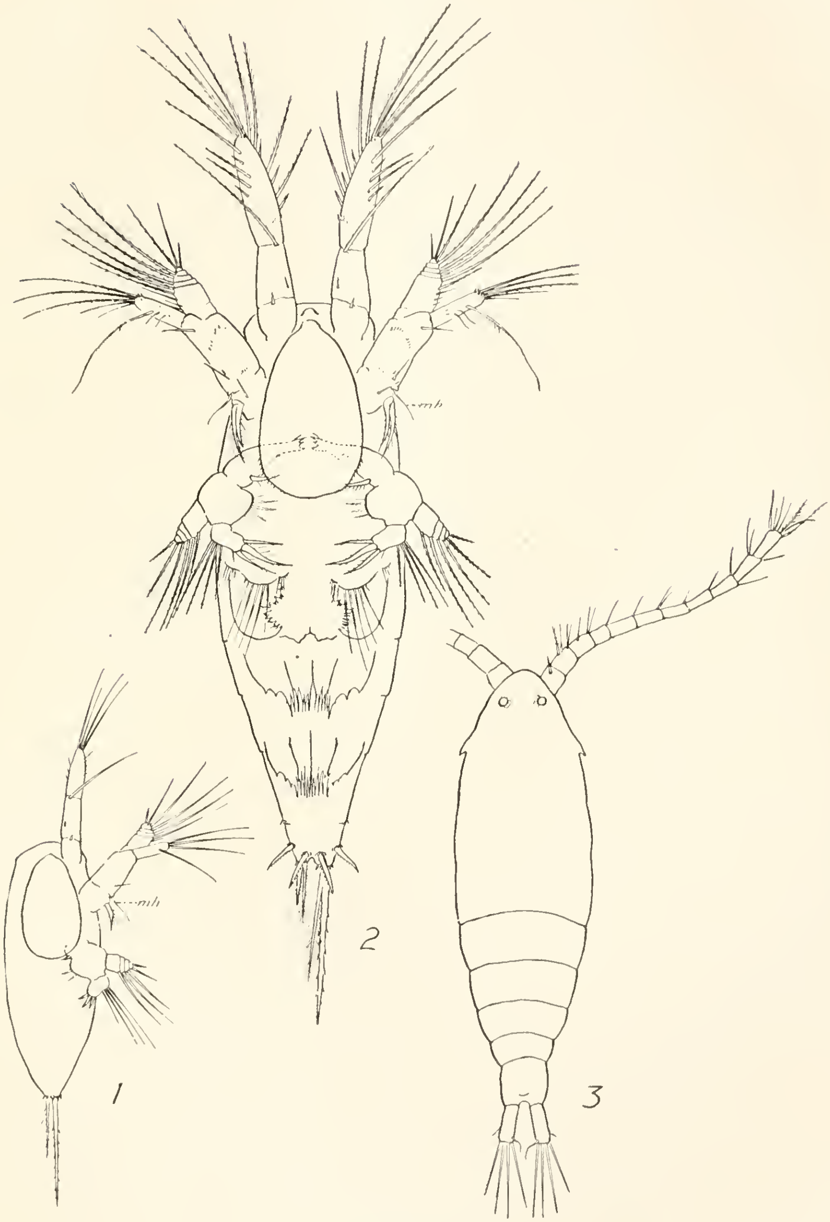
Length 0.424–0.495 mm. (average of eleven measurements, 0.472 mm.).

In this stage there appears an additional short dorsal marginal seta on the distal segment of the first antenna, bringing the total number of dorsal marginal setæ in *L. jollæ* to seven, whereas in *L. trispinosa* the total number in this stage is six, the same as in the fifth nauplius stage.

The Copepodid Stages

In the first copepodid stage of *Labidocera jollæ*, the cephalic hooks and also the rostral processes are wanting. Hence in this stage it is

PLATE III



Labidocera jollæ

FIG. 1. Nauplius Stage II.
FIG. 2. Nauplius Stage VI.
FIG. 3. Copepodid Stage II, dorsal.
Abbreviation: *mh*—masticatory hook of second basipod.

practically indistinguishable from the same stage in *L. trispinosa* except for small differences occurring in the mandibular blade and in the endopod of the maxilliped. In the former species the mandibular blade bears six teeth as compared with only four in the latter species, and in the former the terminal segment of the endopod of the maxilliped bears but two smooth setæ, while in the latter it bears three smooth setæ. These distinctions remain evident through all of the stages, but in Stage II each species acquires an additional tooth on the mandibular blade, giving the total number found in the adult animal (Plate IV, Figs. 11 and 12). In all of the copepodid stages *L. jollæ* is relatively more green in color. The development of the appendages and the body segmentation is the same in both species for corresponding stages.

In the second copepodid stage (Plate III, Fig. 3), *L. jollæ* acquires the rostral processes and also the cephalic hooks. The presence of cephalic hooks serves as a reliable and easy distinction between the species in this and all the following stages.

The sexes are first distinguishable in the fourth copepodid stage by the structure of the fifth legs. The development of these legs is shown for the female copepodid IV and VI in Plate IV, Figs. 17 and 18 respectively. In Stage V, not figured, the legs are almost identical with those shown for Stage VI. In Plate IV, Figs. 19 to 21, is given the development of the male fifth legs from Stage IV to VI.

Explanation of Plate IV

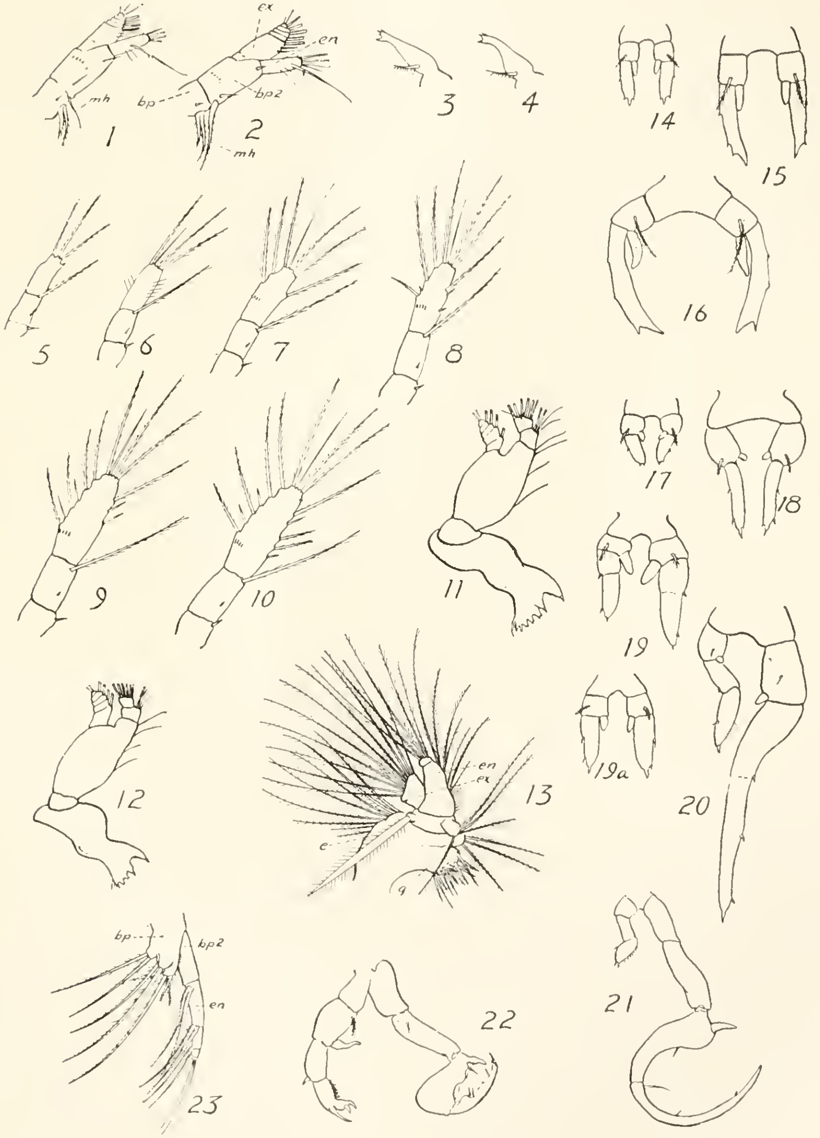
Labidocera trispinosa and *L. jollæ*.

Fig. 1. *L. jollæ*, second antenna.
Fig. 2. *L. trispinosa*, second antenna.
Fig. 3. *L. jollæ*, chewing process.
Fig. 4. *L. trispinosa*, chewing process.
Figs. 5–10. *L. trispinosa*, first antenna nauplius Stages I–VI.
Fig. 11. *L. jollæ*, adult mandible.
Fig. 12. *L. trispinosa*, adult mandible.
Fig. 13. *L. jollæ*, adult first maxilla.
Fig. 14. *L. trispinosa*, female fifth legs, copepodid Stage IV.
Fig. 15. *L. trispinosa*, female fifth legs copepodid Stage V.
Fig. 16. *L. trispinosa*, female adult fifth legs.
Fig. 17. *L. jollæ*, female fifth legs, copepodid Stage IV.
Fig. 18. *L. jollæ*, female adult fifth legs.
Fig. 19. *L. jollæ*, male fifth legs, copepodid Stage IV.
Fig. 19*a*. *L. trispinosa*, male fifth legs, copepodid Stage IV.
Fig. 20. *L. jollæ*, male fifth legs, copepodid Stage V.
Fig. 21. *L. jollæ*, male adult fifth legs, (reduced scale).
Fig. 22. *L. trispinosa*, male adult fifth legs, (reduced scale).
Fig. 23. *L. trispinosa*, adult maxilliped.

Abbreviations: *bp*—first basipod
bp2—second basipod
e—epipod
en—endopod
ex—exopod
g—gnathobase
mh—masticatory hook of second basipod

A comparison of body sizes shows that during the nauplius and early copepodid stages, *L. jollæ* is slightly larger than *L. trispinosa*. In copepodid Stages IV and V the measurements are about equal, but in the adult stage the latter is a little longer.

Plate IV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19a
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bp
bp2
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23

The adult of *L. jollæ* agrees essentially with the original description given by Esterly (1906, p. 74).

Female.—Length 2.49–2.67 mm., (average of nine measurements, 2.57 mm.). In this stage the urosome is very asymmetrical (Plate V, Fig. 8), an asymmetry which is first indicated only in the broadening of the right caudal ramus of Stage V (Plate V, Fig. 7).

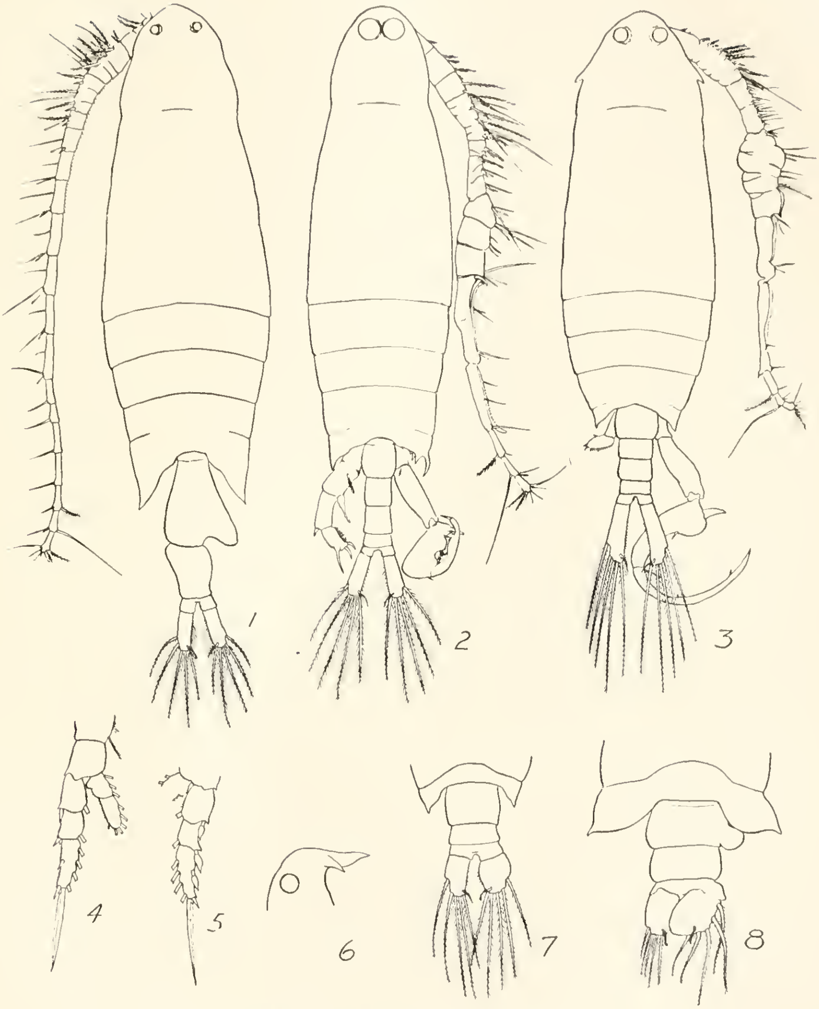
Male.—Length 2.06–2.39 mm. (average of twenty-eight measurements, 2.20 mm.). The rostrum is asymmetrical due to the right prong being much reduced (Plate V, Fig. 6). Esterly also noted this asymmetry but was not certain if it should be considered an individual deformity or a distinct character, since his description was based on a single specimen. I have examined many adult males and find this peculiar asymmetry to be constant in every specimen examined. In the fifth copepodid stage the male rostrum is, however, symmetrical. Another unusual asymmetry found only in the adult male is evident in the modification of the outer spines occurring on the exopod of the right first leg (Plate V, Fig. 5). These are broader and more leaf-like than the regular outer spines found on the other swimming legs (Plate V, Fig. 4).

REMARKS

It has been shown by different workers that the nauplius larvæ of marine copepods belonging to the same genus are, as one might expect, strikingly similar, and may in some instances be identical, as is shown by Oberg (1906) and Gurney (1931) for *Acartia longiremis*, *A. bifilosa*, and *A. clausi*.

In view of the great likeness found in the larvæ of related copepods, the two larvæ herein described are doubtless typical of the genus *Labidocera* and, perhaps with some modifications, of the whole family *Pontellidæ*. Thus far, however, we have information on only three genera of the group, namely, *Labidocera*, *Epilabidocera* (syn. *Paralabidocera*), and *Pontella* (incomplete). The larvæ of the present species of *Labidocera* are very similar to the corresponding stages of *Epilabidocera amphitrites* recently described (Johnson, 1934*b*), and judging from the few figures given by Claus (1893) for *Pontella mediterranea* there appears to be here also essential agreement as to type. An elongated body is common to the three genera, though much accentuated in *Pontella*. The arrangement and type of caudal armature is the same in the investigated species of *Labidocera* and *Epilabodocera*, and agrees with *P. mediterranea* but for the exaggerated size of the left terminal spine in the latter. The first antennæ of the three genera agree as to segmentation and shape and the armature of

PLATE V



Labidocera trispinosa and *L. jollæ*

FIG. 1. *L. trispinosa*, adult female, dorsal.
FIG. 2. *L. trispinosa*, adult male, dorsal.
FIG. 3. *L. jollæ*, adult male, dorsal.
FIG. 4. *L. jollæ*, adult male left first leg.
FIG. 5. *L. jollæ*, adult male exopod right first leg.
FIG. 6. *L. jollæ*, adult male head, lateral.
FIG. 7. *L. jollæ*, female copepodid V posterior end, dorsal.
FIG. 8. *L. jollæ*, adult female posterior end, dorsal.

TABLE I

Identification Table for Nauplius Stages of Labidocera trispinosa and L. jollæ

Stage		I	II	III	IV	V	VI
Length in mm.	*L. trispinosa*	0.130–0.140	0.175–0.201	0.215–0.271	0.250–0.325	0.310–0.370	0.360–0.420
	L. jollae	—	0.200–0.211	0.260–0.270	0.265–0.319	0.390–0.426	0.424–0.495
First Antenna distal segment	*L. trispinosa*	3 terminal setae	4 terminal setae, 1 dorsal and 1 ventral series of fine hairs	4 terminal setae, 2 dorsal and 1 ventral marginal setae (fine hairs wanting)	As in III, but 4 dorsal and 3 ventral marginal setae	As in IV, but 6 dorsal and 3 ventral marginal setae	As in V, but 6 dorsal and 5 ventral marginal setae
	L. jollae	—	As above	As above	As above	As above	As above, but 7 dorsal and 5 ventral marginal setae
Caudal Armature		2 small end spines	1 long end spine and 1 shorter end seta	1 long and 1 short end spine, 2 end setae, and 1 pair ventral hooks	As in III	As in IV, but also 1 pair lateral hooks	As in V
First Maxilla		0	0	Bud	Bud	Rudimentary	Rudimentary
Second Maxilla		0	0	0	Bud	Bud	Rudimentary
Maxilliped		0	0	0	0	Bud	Bud
First and second legs		0	0	0	0	0	Rudimentary

TABLE II

Identification Table for Copepodid Stages of Labidocera trispinosa and L. jollæ

Stage		I	II	III	IV	V	VI
Length in mm.	*L. trispinosa*	0.498–0.590	0.720–0.797	1.03–1.11	1.34–1.49 ♀ 1.23–1.41 ♂	2.04–2.06 ♀	2.50–2.82 ♀ 2.14–2.47 ♂
	L. jollae	0.570–0.650	0.855–0.990	1.20–1.215	1.34–1.50 ♀ 1.35–1.43 ♂	1.97–2.00 ♀ 1.71–1.78 ♂	2.49–2.67 ♀ 2.06–2.39 ♂
Head	*L. trispinosa*	Rostral processes and cephalic hooks wanting	Rostral processes present, cephalic hooks wanting	As in II	As in III	As in IV	As in V
	L. jollae	Rostral processes and cephalic hooks wanting	Rostral processes and cephalic hooks present	As in II	As in III	As in IV	As in V
Thoracic Segments		4	5	6	6	6	6
Abdominal Segments		1	1	1	2	2 ♀ 3 ♂	2 ♀ 4 ♂
Mandibular teeth	*L. trispinosa*	4	5	5	5	5	5
	L. jollae	6	7	7	7	7	7
Legs present		1st and 2nd (3rd rudimentary)	1st, 2nd, and 3rd (4th rudimentary)	1st, 2nd, 3rd and 4th (5th rudimentary)	1st, 2nd, 3rd, 4th and 5th (modified)	As in IV	As in V

at least the species of *Labidocera* and *Epilabidocera* is essentially the same. The number of ventral marginal setæ is the same in all three species, but *E. amphitrites* possesses a total of eight dorsal marginal setæ, as opposed to seven in *L. jollæ* and six in *L. trispinosa*. An alternation of long and short setæ in the dorsal marginal series is common to all three species. It is also characteristic of the living larvæ of all three species to hold the first antennæ extended straight forward and in contact when not swimming.

In Tables I and II are given the characters most useful in identification of the various nauplius and copepodid stages.

Summary

1. The developmental stages of *Labidocera trispinosa* and *L. jollæ* are described and figures given.
2. Each species passes through the typical six nauplius and six copepodid stages.
3. It is probable that in the first nauplius stage the species are indistinguishable.
4. In the adult condition the species are sharply distinguished, but during the second to sixth nauplius stages and during the first copepodid stage they are separable only by small but yet definite specific characters, the most useful of these being the type of masticatory hook occurring on the second basipod of the second antenna.
5. Specific identification of the nauplius larvæ was established by experimentally rearing the sixth nauplius stage through metamorphosis.
6. Tables I and II are given to facilitate identification of the nauplius and copepodid stages.
7. The nauplius larvæ of *Labidocera* are very similar to the larvæ of *Epilabidocera* and are believed to typify the nauplius larvæ of at least other nearly related genera of Pontellidæ.

BIBLIOGRAPHY

Campbell, M. H., 1934. The Life History and Post Embryonic Development of the Copepods, Calanus tonsus Brady and Euchaeta japonica Marukawa. *Jour. of the Biol. Board of Canada*, **1**: 1.

Claus, C., 1893. Ueber die Entwicklung und das System der Pontelliden. *Arb. Zool. Inst. Wien u. Zool. Sta. Triest*, **10**: 233.

Esterly, C. O., 1905. The Pelagic Copepoda of the San Diego Region. *Univ. Calif. Publ. Zool.*, **2**: 113.

Esterly, C. O., 1906. Additions to the Copepod Fauna of the San Diego Region *Univ. Calif. Publ. Zool.*, **3**: 53.

Esterly, C. O., 1912. The Occurrence and Vertical Distribution of the Copepoda of the San Diego Region, with Particular Reference to Nineteen Species. *Univ. Calif. Publ. Zool.*, **9**: 253.

GIBBONS, S. G., 1933. A Study of the Biology of Calanus finmarchicus in the North-western North Sea. Fisheries, Scotland, Sci. Invest., No. 1, 1–24.

GURNEY, R., 1934. The Development of Rhincalanus. *Discovery Reports*, **9**: 207.

JOHNSON, M. W., 1934a. The Life History of the Copepod Tortanus discaudatus (Thompson and Scott). *Biol. Bull.*, **67**: 182.

JOHNSON, M. W., 1934*b*. The Developmental Stages of the Copepod Epilabidocera amphitrites McMurrich. *Biol. Bull.*, **67**: 466.

LEBOUR, M. V., 1916. Stages in the Life History of Calanus finmarchicus (Gunnerus), Experimentally Reared by Mr. L. R. Crawshay in the Plymouth Laboratory. *Jour. Mar. Biol. Ass., N.S.*, **11**: 1.

MURPHY, H. E., 1924. Preliminary Quantitative Study of Marine Zooplankton at La Jolla, California. *Ecology*, **5**: 283.

NICHOLLS, A. G., 1934. The Developmental Stages of Euchaeta norvegica, Boeck. *Proc. Roy. Soc. Edinb.*, **54**: 31.

OBERG, M., 1906. Die Metamorphose der Plankton-Copepoden der Kieler Bucht. *Wiss. Meeresunters. Komm. wiss. Untersuch. deutsch. Meer in Kiel, N. F.*, Abt. Kiel, **9**: 37.

THE MORPHOLOGY OF GONYOSTOMUM SEMEN FROM WOODS HOLE, MASSACHUSETTS

FRANCIS DROUET AND AARON COHEN

(From the Departments of Botany, Marine Biological Laboratory, University of Missouri, and Harvard University)

The rarely observed flagellate, *Gonyostomum Semen* (Ehrenb.) Diesing,[1] was collected during July, 1934 in a plankton haul from a sphagnum swamp near Woods Hole, Massachusetts, by Miss Hannah T. Croasdale. The swamp is one of a number of sphagnum bogs found on Cape Cod and the nearby islands. Peculiar to it is a dense growth of the white cedar, *Chamæcyparis thyoides* (L.) BSP., various species of Ericaceæ, etc., which extend throughout the broad shallow margin to the edge of a pond in the center. The locality is commonly referred to by residents of Woods Hole as the Cedar Swamp. It is situated about a quarter of a mile east of the village on Nobska Road.

The waters of the pond appear brownish even in thin layers in transmitted light and are almost black in reflected light. Miss Croasdale, who made monthly pH determinations during the past four summers (1931–1934) in open water in various parts of the swamp, says that the pH remains consistently between 4.4 and 4.6. Our own determinations made during the summer of 1934 lie within this range. The bottom of the pond is covered with decomposing vegetable debris in a finely divided state, so that even when slightly agitated the water becomes opaque. The southern part of the pond, where our collections were taken, is shaded for most of the day by the dense growth of white cedar, etc.; little direct sunlight reaches this part of the lake except for a few hours at midday. Collections taken early in the morning (seven o'clock Standard Time) invariably contained so many individuals of *Gonyostomum* that the brownish water became green in the net and collecting bottle. Collections taken at noon or thereabout brought few individuals of *Gonyostomum*, but many rotifers and trachelomonads. These observations led us to suppose that the organisms are heliophobic and that a diurnal migration takes place to and from the deep waters according to the intensity of the sunlight which strikes the surface. Lemmermann (1910), on the other hand, describes *Gonyostomum Semen* as 'photophilic.' Further observations are necessary to clear up this matter.

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[1] GONYOSTOMUM SEMEN (Ehrenb.) Diesing, *Sitz.-ber. k. Akad. Wiss. zu Wien*, **52**: 332. 1865.

Monas Semen Ehrenberg, *Ber. Verh. k. preuss. Akad. Wiss. in Berlin*, **1853**: 191. 1853.

Rhaphidomonas Semen (Ehrenb.) Stein, Organismus der Infusionsthiere 3 (1). Taf. XIII, Fig. 6–12. 1878.

Since botanical nomenclature among the flagellates begins with Linnaeus' *Species Plantarum*, 1753, according to the decisions reached by the Third International Botanical Congress (1910), we must accept the earliest valid name, *Genyostomum* Diesing, if we are to keep the group separate from the genus *Monas* Ehrenb.

Collections were taken by pouring approximately five gallons of the swamp water, uncontaminated with decomposed material from the bottom, through a net of No. 20 silk bolting-cloth and straining until 200–300 cc. of the liquid remained. This quantity was poured into wide-mouthed, unstoppered bottles and taken at once to the laboratory. When placed in diffused light, the organisms lived in an apparently healthy condition for two or three weeks. By the end of this period, either the rotifers had consumed all of the individuals of the culture or other changes in the medium became so pronounced that the remaining *Gonyostomum* cells burst or encysted. In these cultures in the laboratory, division and, later, encystment of the vegetative cells were observed.

The literature is chiefly vague and non-committal concerning the details of morphology and physiology of *Gonyostomum Semen*, as likewise of other members of the Chloromonadophyceæ (Chloromonadida) with the possible exception of *Vacuolaria virescens* Cienk. (Cienkowsky, 1870; Senn, 1900; Bütschli, 1883). Perhaps this lack of information is due to the apparent rarity of the organisms, which have been reported infrequently since their first recognition. The actual localities for which we find our species previously recorded are three: a sphagnum bog near Berlin (Ehrenberg, 1853; Stein, 1878), a bog near Fölisö in Finland (Levander, 1894), and 'Sphagneten' near Seefeld and Larss in the Tyrol (Dalla Torre and Sarnthein, 1901). Pascher (1913) says of its distribution: "In stehenden Gewässern, Tümpeln und Torfsümpfen; verbreitet, doch vereinzelt." Even if others have observed the organism at other stations, Ehrenberg, Stein, and Levander appear to be the only workers who have contributed to our previous knowledge of the species.

These authors, and compilers of works on the flagellates after them, disagree on certain important details of morphology and physiology of *Gonyostomum Semen*. The flagella, for example, are described variously. Ehrenberg (p. 191) says ". . . ciliis pluribus vibrante." Diesing (1865), who obviously did not see the organism himself, remarks of the genus, "flagello ignoto." Stein mentions and figures two flagella, one projected forward and one trailing.

Levander figures only the forward-projecting flagellum and does not commit himself as to the presence of a trailing one. Similar uncertainty exists concerning the long slime-threads produced from trichocyst-like rods embedded in the cytoplasm. Lemmermann and Pascher place upon Levander all responsibility for the idea that the slime-threads become several times the length of the body of the organism. We find the following statement in Ehrenberg's original diagnosis: "Facile diffluendo ovula glandulam et spiculas bacillares tenues ostendit." It is probable, as Kent (1880) has pointed out, that Ehrenberg's phrase "ciliis pluribus" quoted above refers to the slime-threads when only partially discharged. Likewise, the literature is vague or contradictory as to the function of the triangular cavity and the nature and points of discharge of the contractile vacuoles. Cell division and encystment have not heretofore been described.

Morphology of the Vegetative Cell

In shape and size, the vegetative cells of *Gonyostomum Semen* actually vary more widely than other authors intimate. Generally speaking, the cell body (Figs. 1 and 2) is ovoid and somewhat flattened dorsoventrally, with a short, cylindrical, more or less pointed caudus at the posterior end. The dorsal surface may be ovate, obovate, ovate-lanceolate, obovate-lanceolate, or almost lanceolate or circular in outline, 1–2.5 times as long as broad in our material. In contour, the dorsal surface is convex, often with some irregularities. The ventral surface is similar, except that a shallow longitudinal furrow extends from the opening of the triangular cavity to the posterior region (Fig. 2). This furrow may be lacking in the more flattened and nearly circular types, as also in the extremely lanceolate types. In side view, the organism appears lanceolate to linear-lanceolate, with the widest part near the anterior end. Material examined an

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Explanation Plate I

Fig. 1. Vegetative cell of *Gonyostomum Semen*, ventral surface, showing the two flagella, the chromatophores, the triangular cavity, the trichocyst-like rods, and the position of the nucleus. × 944.

Fig. 2. Diagrammatic representation of side and ventral views of the vegetative cell. The ventral groove and positions of flagella are indicated. × 944.

Fig. 3. Diagrammatic drawing of a cell mounted in 1 per cent acid fuchsin, showing the slime threads. × 377.

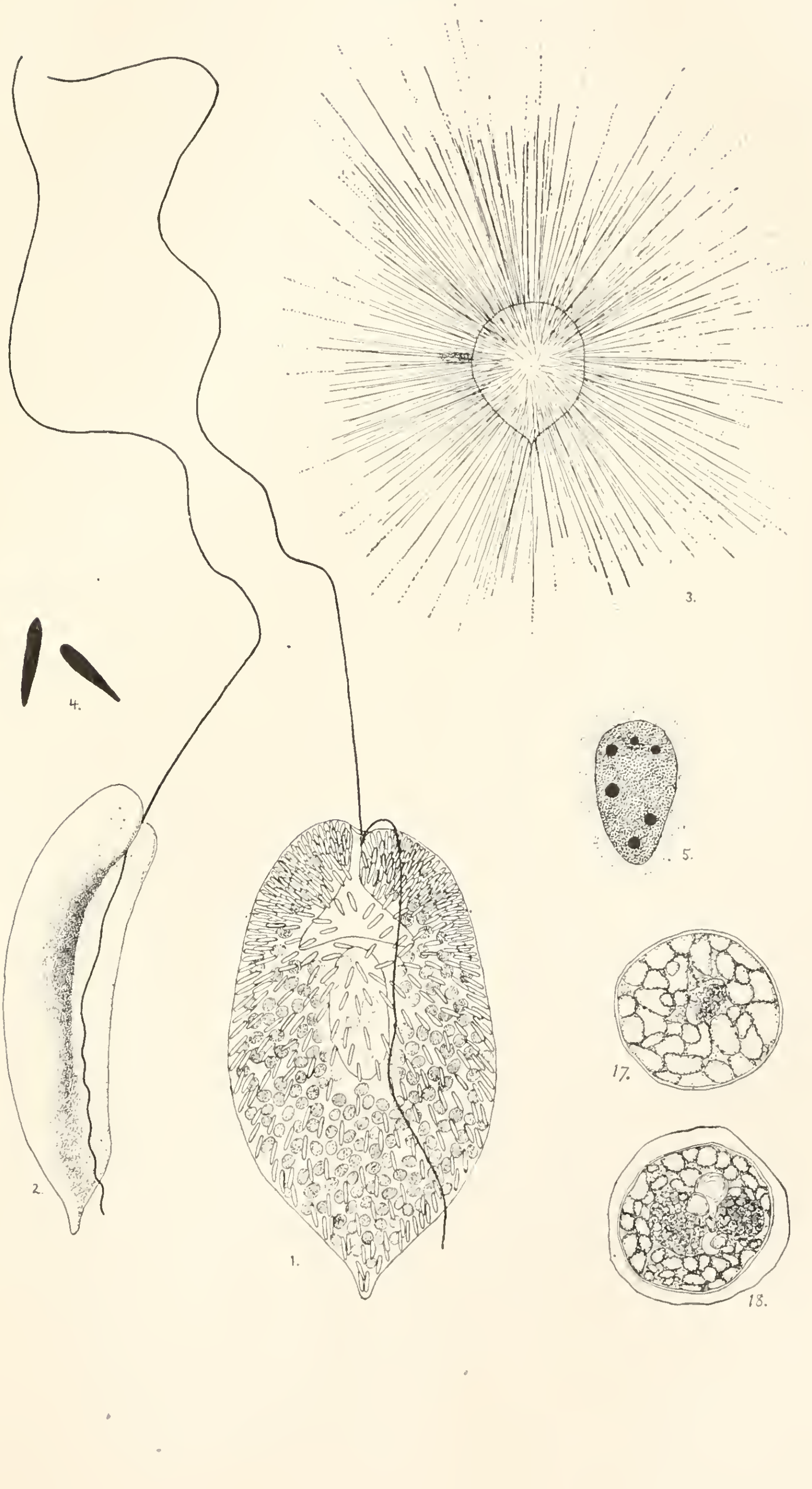
Fig. 4. Trichocyst-like rods discharged into the medium when the cell is mounted in very dilute aqueous acid fuchsin. × 2833.

Fig. 5. Nucleus as stained with cotton blue by Maneval's technique. The large endosome-like bodies and deeply-staining granular matrix are shown. × 944.

Fig. 17. Young cyst, showing the thin gelatinous sheath. × 708.

Fig. 18. Older cyst, with thick sheath, few chromatophores, and much oil. × 708.

Plate I



hour after collection from the Cedar Swamp exhibited as a rule an abundance of the more lanceolate types (as seen in dorsal view); after a day or two of standing in the swamp water cultures in the laboratory, the organisms became more flattened and consequently broader in dorsal outline, so that almost circular cells were often observed; and the lanceolate types were indeed rare. In dorsal view, as also in ventral view, the anterior end is somewhat two-lipped, with the opening of the triangular cavity between the lips. Levander notes that the left lip (as seen in ventral view) is higher than the right lip, an observation which holds true in our material. Under certain external conditions, the caudus disappears entirely; and an organism the shape of *Vacuolaria virescens* results. Under pressure, or in viscid media such as 0.5 per cent agar or quince jelly as prepared by Turner (1917), the organisms become amœboid (Fig. 6). From the above description, it is obvious that the cell has no definitely fixed shape as has *Chlamydomonas* or *Peridinium;* the organism may be described as metabolic, with an ability to change shape in response to a change in the environment similar to that of *Euglena* or *Peranema*.

Ehrenberg's original diagnosis of *Monas Semen* describes the organism as having a length of 1/48 line (*ca.* 45 μ). Levander cites the length as 80 μ and the greatest breadth as 34 μ. Stein, and subsequently Kent, Lemmermann, and Lindau and Melchior (1926) say 44–63.5 μ. In one hundred random measurements of individuals made on various cultures during our observations, we have found that the size varies far beyond the above described limits. The largest individuals observed in our material measured 92 μ × 69 μ; the smallest 36 μ × 23 μ. Among these hundred cells, approximately three-fourths of the measurements lay between 40–80 μ × 30–60 μ. Of the entire number the average length was 62.5 μ and the average width 41.4 μ. It is probable that under environmental conditions other than those to which the cells were subjected by us the extremes of size may be greater than our measurements indicate.

The nucleus, though obscured by other cell contents in the living cell, is easily observed, when the organism bursts, as an ovoid or subglobose body about one-fourth the length of the cell. It is found (in stained preparations) in the center of the cell, though sometimes to one side of the center or definitely in the posterior region. Stein figures the nucleus as containing a single deeply-staining body (the endosome) in its center surrounded by a well-delimited vesicle containing granular chromatic material. Levander states that the nucleus contains such chromatic filamentous structures as are seen in *Peridinium*. Our material, killed in 0.5 per cent osmic acid, preserved in

2 per cent formalin solution, fixed in very dilute chromo-acetic acid, and stained in Delafield's hæmatoxylin, shows the nucleus as a well-delimited vesicular body containing several to many almost spherical, deeply-staining bodies, each 1.5 μ or less in diameter, distributed through a coarsely granular matrix. No one or two of these can be distinguished from the rest as true endosomes, such as Stein figures for *Gonyostomum Semen* and Bütschli describes in *Vacuolaria virescens.* Slides prepared by Dr. W. E. Maneval from the material fixed in osmic acid and preserved in formalin, and stained as temporary mounts [2] with cotton blue or as semi-permanent mounts with a mixture of cotton blue and acid fuchsin, demonstrate this structure even better (Fig. 5). It is not improbable, however, that the formalin produces anomalous granules in the nucleus. In material which is dried on the slide before staining, the detailed structure of the nucleus is often obscured by the overlying chromatophores and trichocyst-like rods. No evidence of a filamentous structure of the nucleus was seen. No material was sectioned and stained.

The cytoplasm, as seen in the living cell, is hyaline, finely granular, and without evident vacuolar structure. In the periphery of the cytoplasm and sometimes throughout the mass, lie the contractile vacuoles, the chromatophores, and the trichocyst-like rods. The outer layers, in the living state, exhibit no conspicuous alveolar structure as that which Senn describes and figures in *Vacuolaria virescens.* Yet, when a cell bursts or is stained without preliminary drying, as with Maneval's stains described above, the cytoplasm in which the chromatophores etc. are embedded appears less viscous and less granular than that immediately surrounding the nucleus.

At the anterior end of the organism, a duct opening to the outside leads into a cavity in the cytoplasm usually described as triangular in optical longitudinal section (the 'dreieckige Blase' of Lemmermann and of Pascher). Oltmanns (1922) describes this cavity as "von der Form eines breiten Kegels (sie erscheint im Schnitt dreiseitig)." In our material the cavities are somewhat flattened dorsoventrally and vary in shape from one-third as wide as long (in ventral view of the cell) to twice as wide as long. The function of this cavity is not as yet clearly understood. Morphologically, the triangular cavities of *Gonyostomum Semen* and of *Vacuolaria flagellata* (Stokes) Senn (Stokes, 1886 and 1888; Senn, 1900) may be homologous with the so-called 'flagellar pits' of *Thaumatomastix setifera* Lauterb. and of *Vacuolaria virescens* Cienk. (Lauterborn, 1899; Cienkowsky, 1870; Bütschli, 1883; Senn, 1900). In the works on the flagellates cited at the end of this paper, one is given the impression that the contractile vacuoles discharge into this cavity.[3] Various observations of our own, insufficient at present for definite conclusions, indicate that this supposition is plausible. However, other observations indicate that the contractile vacuoles discharge through the outer cell membrane. The development of triangular cavities in dividing cells is described below.

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[2] The material is mounted directly in the following solution (Maneval): 15 parts of 5 per cent phenol, 4 parts of glacial acetic acid, and 1–3 parts of a 1 per cent aqueous solution of cotton blue. For the semi-permanent mounts, a modification of Amann's lacto-phenol (Maneval) was used: 20 grams of phenol, 20 cc. of lactic acid, and 40 cc. of glycerine are dissolved in 20 cc. of water. To this 20 per cent by volume of glacial acetic acid is added. Finally, equal parts of 1 per cent aqueous cotton blue and 1 per cent aqueous acid fuchsin are added to the mixture in quantities suitable for the type of staining preferred.

One large contractile vacuole, said by Levander to contract about once every minute, is present in the cytoplasm somewhere in the vicinity of the triangular cavity. Levander places it "am Ende des Geisselkanals," though in our material it is more often found nearer the outer cell membrane than the cavity. Smaller contractile vacuoles were observed in some individuals scattered through the anterior cytoplasm. These appear to coalesce sooner or later and finally to empty into the large anterior vacuole. Whether the large vacuole discharges at length through the outer cell membrane or into the triangular cavity, we cannot at present be certain.

The chromatophores are small ovoid bodies of a peculiarly bright green color—the *maigrün* of German authors. As in other chloromonads, the chromatophores of *Gonyostomum Semen* become a dull blue-green in color when treated with a dilute acid, *e.g.*, 5 per cent HCl, a reaction (Pascher, Senn) indicative of the presence of an excess of xanthophyll. The chlorophyll (Pascher) is thought to be of a chemical composition somewhat different from that encountered in other flagellates and green plants. The chromatophores are arranged peripherally in the cytoplasm over the entire body of the organism with the exception of the caudus. Red-pigmented bodies (eye-spots, stigmata) are apparently absent.

No starch or starch-like substances which produce a blue color when treated with iodine were found in the cells. Oil occurred as slightly amber-colored globules distributed through the cytoplasm of cells observed shortly after collection from the Cedar Swamp. As the laboratory cultures became older, the oil droplets grew larger and more conspicuous, especially in the broader and more flattened individuals. Often one or two large irregular oil bodies comprised half the length of the cell and measured one-third to one-half as wide as long in optical section. Since these conspicuous droplets were not seen in small individuals which showed evidence of recent or future division, it is supposed that they indicate a pathological condition of the protoplasm. Similar production of excess oil under unfavorable cultural conditions was observed by Bohlin (1897) in *Chloramœba heteromorpha*. Treatment with 0.5 per cent osmic acid for a week or more at room temperature blackens the oil droplets entirely. A saturated alcoholic solution of Sudan III causes the cells to burst and allows the droplets to lie free in the medium; these droplets absorb the Sudan III readily. When organisms killed in the fumes of osmic acid are allowed to dry on the slide, the oil globules are easily discerned in the dried cells. If a drop of xylene or Canada balsam is placed on the material, the oil globules disappear. Cells fixed by other agents likewise lose the oil droplets when mounted in xylene or balsam. Levander records the finding of paramylon [4] in the cells of *Gonyostomum Semen*. He gives us no reason to suppose that he investigated the chemical nature of these bodies in the painstaking manner employed by Bütschli (1906) on the paramylon bodies of certain Euglenophyceæ.

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[3] Oltmanns speaks of the function of the cavity: "Sie ist selber nicht kontraktil, steht aber mit pulsierenden Vakuolen in Verbindung, die wohl in sie einmünden. Die Sache erinnert an Euglenen und an Peridineen." Likewise, Senn remarks: "Bei anderen Formen . . . (Rhaphidomonas und Thaumatomastix) hat sich eine constant vorhandene, nach aussen offene, nicht mehr pulsierende Hauptvacuole ausgebildet, in welche sich die seitlich entstehenden Nebenvacuolen abwechselnd entleeren (Fig. 125)." The figure cited illustrates the vacuolar system of *Thaumatomastix*, redrawn from Lauterborn.

Two flagella are present, each as long as, or longer than, the body of the organism. The one projected forward (tractellum) is attached to the inner wall of the duct leading from the triangular cavity. The insertion of the trailing flagellum (pulsellum) has not been determined; however, if it were inserted on the wall of the duct its basal portion would surely have been seen there. It is probably attached to the cell membrane near the anterior end of the ventral groove, as Stokes has recorded in *Vacuolaria flagellata*. As Levander has already noted, the organism progresses forward in a slow, spiral course, the basal portion of the tractellum projected directly in advance, moving little if at all, the apical portion spiralling so rapidly that it is invisible until the organism has come to rest. Then, when the cell is quiescent, such as following the addition of very dilute acid fuchsin (1 drop of 1 per cent aqueous acid fuchsin in 50 cc. water) to the mount, both flagella can be observed. The pulsellum was never seen in its entirety and is therefore shown in Fig. 1 as shorter than the tractellum. In the dilute acid fuchsin, the vibratile portion of the tractellum lashes back and forth in slow spirals; the pulsellum often leaves the ventral groove and is projected outward or forward. The attenuated apical portion of the tractellum was seen also by carefully focusing the oil-immersion objective when dark-field illumination was employed. Since the apical parts of the flagella are so delicate, an almost perfect dark field, free from foreign particles in the mount and from scratches on the glassware, is necessary. It was found impracticable to stain the flagella, since the gelatinous threads discharged from the trichocyst-like rods obscure the flagella when a stain is applied. Since no microtome sections were made, basal granules and other structures of a kinetic apparatus remain unidentified.

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[4] To quote (p. 34): "An der Dorsalseite des Vorderendes sah ich öfters einige ganz minimale, ringförmige Körner, die wohl Paramylum-körner waren."

No cell wall is present about the organism. The cell has, as a rule, a definite shape as has *Euglena;* only under certain marked departures from the usual conditions found in the medium, however, does the shape change as rapidly as does that of *Euglena*. The protoplast itself is delimited from the surrounding medium by a very thin plasma membrane, which does not separate from the rest of the cytoplasm when the organism is placed in concentrated salt solutions like chlor-zinc-iodine (Nowopokrowsky, 1911), as does a cell wall. No blue or violet color appeared during treatment with this reagent to indicate the presence of cellulose or related carbohydrates in the membrane. When the liquid beneath the cover-slip dries slowly so that the organisms are subjected to gradually increasing pressures, the cells become amœboid. Under such pressures, a definite and distinct line (or membrane) can be seen delimiting the outer cytoplasm of each lobe. Sooner or later, if the pressure continues to be increased, the membrane breaks; and the organism bursts beyond recognition. While the membrane is still intact, the flagella move slowly or remain stationary; if more water is added to the mount (and the pressure thereby diminished), the cell regains its usual shape and the flagella resume normal activity. Amœboid cells of *Gonyostomum Semen* were found in a medium containing 0.5 per cent agar in swamp water. In such cultures, the cells lived at least 24 hours with the chromatophores and other cell structures in a healthy condition and with the trichocyst-like bodies undischarged. The lobed condition here is due probably to mechanical pressure exerted by the agar on the surfaces of the cells. Amœboid shapes were likewise assumed by cells in the process of division, as described below. The fact that *Gonyostomum Semen* exhibits such 'metaboly' as do various of the Euglenophyceæ affords further evidence, though indirect, of the absence of a true cell wall.

Distributed radially in the peripheral cytoplasm are rod-shaped bodies (Fig. 1) characterized in the living cell by their ability to absorb large amounts of dye from very dilute aqueous solutions. Ruthenium red, Gram's iodine, acid fuchsin, methylene blue, methyl green, safranin, and Delafield's hæmatoxylin are a few of the dyes absorbed in this manner. As Levander has stated, the rods are arranged in dense 'palisade-like' formation in the anterior half of the cell, and here are most abundant in the lobes on either side of the triangular cavity. Toward the posterior end the distribution is less orderly and less abundant. The objects appear to be least numerous in the caudus, though some individuals have been observed to possess as many as ten of the rod-like bodies here.

When a very dilute solution of a dye (1 drop of 1 per cent aqueous acid fuchsin in 50 cc. water) is added to the mount, a few of the rods burst suddenly through the cell membrane and become elongated into threads of slime as long as or several times longer than the body of the organism. Others come through the cell membrane and lie free in the medium as club-shaped bodies (Fig. 4), rounded at one end and pointed at the other. Others remain half on either side of the membrane, but most remain in their original positions within the cytoplasm. In any case, the bodies quickly absorb the dissolved dye, whether they are inside or outside the cell. When more concentrated solutions of dyes (such as 1 per cent aqueous acid fuchsin) are allowed to diffuse beneath the cover-slip, the rods are discharged suddenly, and the slime-threads produced become deeply stained at once, so that the cell becomes the center of a mass of radiating gelatinous threads (Fig. 3). The threads often appear branched and *en masse* are similar to a much-branched fungus mycelium surrounding the cell, as described by Levander. When discharged in a salt solution, without the addition of a dye, the threads are difficult to see except at their bases, where they have the greatest diameter when stained. If the cells are mounted in chlor-zinc-iodine, the rods do not discharge, as Scherffel (1912) has described in *Monomastix* and *Pleuromastix*, nor do they leave the cell without elongating, as Levander has described in *Gymnodinium*.

A large percentage of the cells treated with 1 per cent solutions of the dyes mentioned above burst when the trichocyst-like bodies are suddenly discharged. It is supposed here that the membrane surrounding the protoplast is broken as the rods or filaments shoot through it. Scherffel has described and figured visible holes in the cell membranes of *Monomastix*, but the rods in that organism are comparatively much larger and less numerous than those of *Gonyostomum*. Often the discharge of the bodies and consequent bursting of the membrane (or *vice versa*) can be caused by pressure. This is demonstrated when a thin film of culture medium containing the cells dries on the slide. The gradually increasing concentration of dissolved materials in the medium may be partially responsible for the bursting or discharge, but similar results are obtained when the organisms dry in distilled water.

The bursting of the cells upon the addition of various dissolved materials introduces the greatest difficulty in handling *Gonyostomum Semen*. Several types of killing and fixing agents were used on the cells with disastrous results: 1 per cent–95 per cent alcohol, $HgCl_2$ (concentrated solution in water and in 50 per cent alcohol), chromo-acetic acid (1–10 drops in 20 cc. water), Flemming's solution, and 1 per cent–3 per cent formalin in water. Inverting a slide containing a drop of the culture solution over the fumes of 1 per cent osmic acid met with the best success. The organisms also were killed satisfactorily when an equal part of 1 per cent osmic acid was added to the medium. Maneval's nuclear stain [5] proved almost as successful as a killing agent. If first killed in osmic acid or Maneval's stain, the cells could be fixed in other reagents without distortion or bursting. Difficulties similar to those encountered in fixing and staining were met with also in transferring from one culture medium to another.

When a collection is taken from the Cedar Swamp and left standing in a jar of swamp water for a day, many of the organisms fall to the bottom of the jar and become aggregated there in a gelatinous stratum. A piece of such a mass under the microscope appears to be composed of cells of the usual vegetative structure lying in a gelatinous matrix. When stained with very dilute iodine or acid fuchsin, the gelatinous material is seen to be composed of mycelium-like threads such as those about organisms from which the trichocyst-like bodies have been discharged because of a chemical stimulus. The cells, upon close inspection, contain few of the rod-like bodies. It is probable that the rods are discharged because of some change in the chemical or physical nature of the medium; the slime-threads of various individuals become enmeshed; the flagella are caught in the gelatinous mass and are rendered functionless; and the organisms sink to the bottom of the jar because of their specific gravity. In the gelatinous mass the penetration of osmic acid and stains is very slow. In the case of stains, at least, the dissolved material is absorbed in large quantities by the outer part of the matrix. Senn (p. 104) gives us the impression that such gelatinous coverings are produced by many flagellates: "Gelegentliche Ausscheidung weicher Gallerte ist bei sehr vielen, besonders mit Chromatophoren versehenen Formen (Euglenaceae, Chloro- und Chrysomonadineae) haüfig. Durch ungünstige Verhältnisse (Druck, Zusätze von Reagenzien) treten aus dem Periplasten geschlangelte Gallertfaden, die durch ihre nachträgliche Verquellung die Zelle in einen losen Mantel einhüllen. Mit dieser gelegentlichen Gallertausscheidung muss auch die Bildung von Gallerthüllen durch Dauercysten in Beziehung gebracht werden." In *Euglena*, Klebs (1883) described the slime-producing bodies as being attached to the cell membrane. In *Gonyostomum Semen*, the bodies do not appear to be attached to the membrane nor do they appear to be attached to the membrane in Levander's figures of *Gymnodinium* or in Scherffel's figures of *Monomastix* and *Pleuromastix*. Whether or not these bodies in the green flagellates can be viewed as true trichocysts such as those described by Mitrophanow (1905) and Schuberg (1905) in *Paramæcium* and other Ciliata remains an open question.

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[5] A variation of the first stain described in footnote 2 of this paper: 60 parts of 5 per cent aqueous phenol, 10 parts of 30 per cent aqueous $FeCl_3$, 20 parts of glacial acetic acid, and 15 parts of 1 per cent aqueous acid fuchsin. The organisms are mounted directly in this solution. If the staining is too deep, the material may be destained to the desired color with Amann's lacto-phenol (the second solution of the same footnote without the dye added).

CELL DIVISION

Many of the smaller individuals found in the free-swimming and the gelatinous conditions described above, upon being treated with nuclear stains, showed the presence of two nuclei within each cell (Fig. 7). Other individuals exhibited 'swallow-tails' (Figs. 8 and 9) at the posterior ends. These peculiar features appeared in the cultures regularly during midday and the early evening. They led us to look further into the matter of cell division.

Hanging-drop cultures of swamp water were constructed and observations made almost continuously during the periods 11:00 A.M. to 1:00 P.M. and 6:30 P.M. to 8:30 P.M. Standard Time. A large number of cells were seen in various stages of division, and the entire process was watched in several individuals. The time required for complete division in the swamp water cultures was regularly 45–55 minutes. If an equal quantity of quince-seed jelly were added to the hanging-drop, the movements were slowed down and the time required for the process lengthened.

Division is longitudinal, as in other wall-less Flagellata, often slightly oblique. The cell enlarges in transverse section, becomes more metabolic and amœboid in form, and loses the flattened shape so characteristic of the vegetative individual. Usually a lobe appears at one side of the caudus, so that two 'tails' are apparent. From each arm of the triangular cavity, as seen in ventral optical section, an extension of the cavity is sent posteriorly and laterally (Fig. 9). At the extremities of these arms, two new arms are pushed out at angles to each other into the cytoplasm (Fig. 11). These enlarge to form two new triangular cavities, each opening into the original one. Accompanying or following the formation of new cavities, a longitudinal furrow extends about the long axis of the cell body, beginning with the constriction between the 'tails' or at the anterior end in a plane bisecting the original triangular cavity longitudinally (Figs. 12–15). No matter at which end it is first evident, the furrow soon becomes conspicuous at both ends and extends rapidly to the central part of the body, ultimately constricting the mother into two daughter cells (Fig. 16). The flagella move vigorously throughout this process, and the entire body of the cell becomes contorted, exhibiting much 'metabolic' activity. New flagella appear soon after the anterior end splits in division (Fig. 14), so that the dividing individual possesses four wildly lashing flagella, seemingly hastening the separation of the cells by pulling in different directions. The new triangular cavities, as may be readily seen in Figs. 11, 12, 13, and 15, are not formed originally at the anterior apices of the constricting cells; rather, they

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EXPLANATION OF PLATE II

FIG. 6. Amœboid cell body produced by pressure of the cover-slip in a gradually drying mount. × 708.

FIG. 7. Two-nucleate cell found in swamp water culture at noon. Fixed in osmic acid and stained with Delafield's hæmatoxylin. × 377.

FIG. 8. Cell in the first stages of division, with 'swallow-tails' at the posterior end. × 377.

FIG. 9. Cell with elongated and 'lobed' triangular cavity and with conspicuous 'swallow-tails.' × 472.

FIG. 10. Cell with first constriction appearing in the anterior region, instead of in the posterior as in Figs. 8 and 9. × 472.

FIG. 11. Amœboid cell in division, showing the triangular cavities of the daughter cells formed at the base of the cavity of the mother cell. × 708.

FIG. 12. The same cell constricting at the anterior end. This constriction appears to bisect the triangular cavity of the mother cell. × 708.

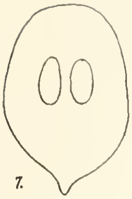
FIG. 13. A later stage in constriction of the same cell. × 708.

FIG. 14. The two daughter cells attached by only a small protoplasmic connection, each cell bearing two flagella. × 708.

FIG. 15. Same as Fig. 14, but showing the two cells with distinct triangular cavities. × 708.

FIG. 16. The same, with the daughter cells pulling apart. × 708.

PLATE II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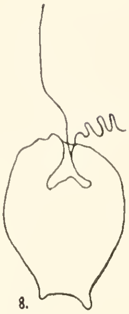
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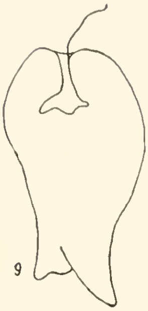
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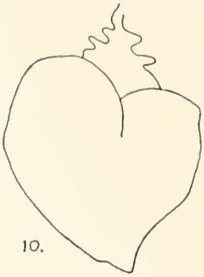
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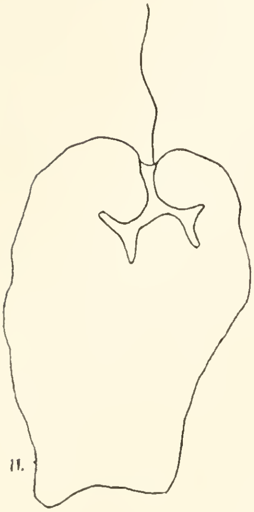
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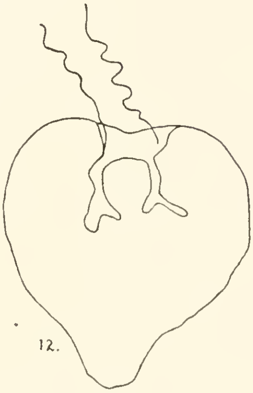
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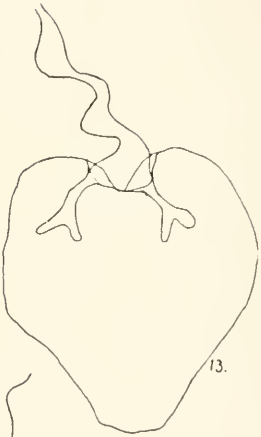
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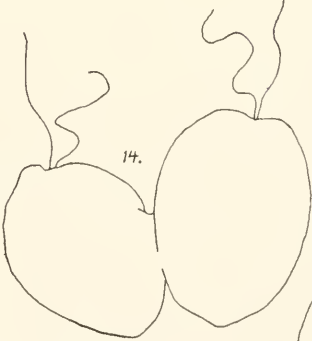
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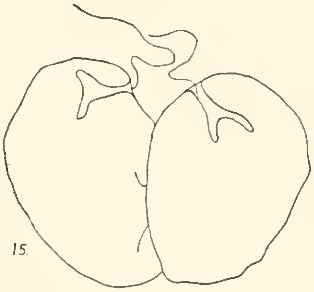
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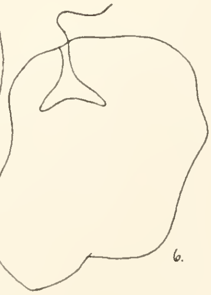
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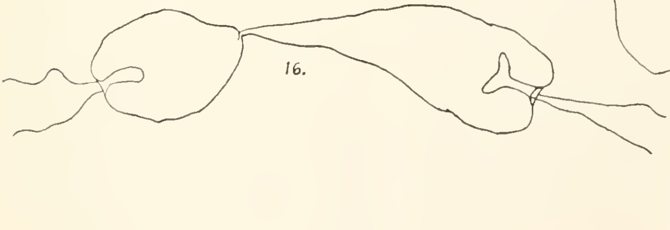
16.

first appear laterally, and by the splitting of the original triangular cavity and by change in shape of the daughter during the later stages of division, are finally (Fig. 16) oriented at the apex. The fate of the flagella of the mother cell and the origin of the flagella of the daughters were not noted.

As pointed out above, the dividing cells were primarily those belonging to the smaller size ranges of the population, though a few large flat cells appearing as the culture aged exhibited 'swallow-tails.' These latter types invariably contained large irregular oil bodies and possibly were pathological, for none were observed to proceed further in division. Such observations, if simulated by future studies in *Gonyostomum Semen*, may provide further evidence in favor of Adolph's (1931) contention that the frequency of division is inversely proportional to protoplasmic growth; in other words, the larger the cells in the population, the smaller the frequency of division.

ENCYSTMENT

Encysted cells appeared in two-day-old hanging-drop cultures of swamp water and also in tightly stoppered swamp-water cultures placed for several days in the dark. In the latter cultures, the encysted material was abundant and lay in yellowish palmelloid masses in the bottom of the liquid. Cultural conditions in these stoppered dishes were obviously unfavorable, for the cysts here exhibited signs of deterioration—the production of much oil and evident disintegration of the chromatophores.

In the hanging-drop cultures, young cysts appeared as spherical bodies 25–36 μ in diameter and surrounded by a thin hyaline sheath (Fig. 17). In some specimens the caudus could be distinguished as an actual projection of the spherical body until the sheath had grown to several microns in thickness. The older cysts (Fig. 18) have a thick gelatinous sheath like that which Cienkowsky (1870) has described and figured for *Vacuolaria virescens*. No trichocyst-like bodies were apparent in these older cysts. Beneath the sheath, the peripheral layer of chromatophores, many oil globules, and several (1–3 in our observations) contractile vacuoles were distinguished. These vacuoles, each measuring 3–4 μ in the diastole, were observed, especially in the young cysts, to discharge directly through the cell membrane. The nucleus was not identified. In old cysts, large clumps of dark brown material appeared in the cytoplasm. The sheath stains intensively with ruthenium red and other dyes, a characteristic which leads us to suspect that pectic substances are present. The sheath obviously prevents the ready passage of dissolved dyes (and other substances?) into the included protoplast.

Division probably occurs in these encysted cells. Our limited time did not permit such observations on this material.

SUMMARY

1. The chloromonad, *Gonyostomum Semen* (Ehrenb.) Diesing, was collected in a sphagnum swamp at Woods Hole, Massachusetts, during July, 1934. The organism has been seen so rarely since it was first described that certain morphological features of the vegetative cells have been imperfectly known, and cell division and encystment have not previously been recorded.

2. The vegetative cells are flattened dorsoventrally and vary in shape in ventral view from lanceolate to circular. The anterior end is two-lobed, and a short caudus terminates the posterior end. No cell wall is present: the cells are somewhat metabolic and under certain conditions become amœboid. The extreme size measurements of a hundred cells were 36–92 μ × 23–69 μ. On the ventral surface, a shallow longitudinal groove runs from anterior to posterior regions. Two flagella are present, one projected forward, and one trailing close to the cell body in the ventral groove. A broadly conical cavity lies in the anterior cytoplasm and opens to the outside by a small aperture between the anterior lobes. A large contractile vacuole is seen near this cavity, and several smaller ones may be present. The nucleus is ovoid or subspherical and usually located centrally in the cell. Its structure has not yet been clearly shown. The chromatophores are small, ovoid, and arranged peripherally in the cytoplasm. Oil is the product of metabolism, and under obviously unfavorable conditions it is formed in large quantities. Many hyaline rod-like bodies are arranged radially in a peripheral layer in the cytoplasm. These bodies absorb large amounts of stains from very dilute solutions. Under pressure, or upon addition of chemicals, these bodies shoot out of the cell and usually elongate into threads of slime. They act like the trichocysts in *Paramœcium* and force the worker to exercise extreme care in fixing, preserving, and culturing the organisms.

3. Division of vegetative cells is longitudinal; it occurs, as a rule, only among the smaller cells of the population. Nuclear division and cytoplasmic constriction are described.

4. Encystment of cells was observed in old cultures. Division of encysted cells was not observed.

We are indebted to members of the Department of Botany, the Service Department, and the Library of the Marine Biological Laboratory, who made this work possible. We wish to express our gratitude especially to Dr. William Randolph Taylor and Dr. W. C. Curtis for advice and criticism during preparation of the manuscript; to Dr. W. E. Maneval for the use of his staining formulæ and for his coöperation in staining material for us; and to Dr. Hannah T. Croasdale and to many others at Harvard University, the University of Missouri, and the Missouri Botanical Garden for numerous valuable services. Specimens of *Gonyostomum Semen* from Woods Hole have been placed in the Farlow Herbarium and the Herbaria of the Marine Biological Laboratory, the University of Missouri, the Missouri Botanical Garden, and the New York Botanical Garden.

LITERATURE CITED

Adolph, E. F., 1931. Regulation of Size as Illustrated in Unicellular Organisms. Baltimore.

Bohlin, K., 1897. Zur Morphologie und Biologie einzelliger Algen. *Öfvers. f. Vet.-Akad. Forhandl. Stockholm.* Reprint.

Bütschli, O., 1883–85. Die Protozoen. In Bronn's "Klassen und Ordnungen der Tierreichs" **1** (2). Mastigophora.

Bütschli, O., 1906. Beiträge zur Kenntniss des Paramylons. *Arch. f. Protist.* **7**: 197.

Cienkowsky, L., 1870. Ueber Palmellaceen und einige Flagellaten. *Arch. f. mikr. Anat.*, **6**: 421.

Congrés international de Botanique, 1910. Actes, Vol. 1. Brussels.

Dalla Torre, K. W., and L. Grafen von Sarnthein, 1901. Die Algen von Tirol, Verarlberg und Liechtenstein. In "Flora der Tirol, Verarlb. u. Liechtenstein," vol. 2. Innsbruck.

Diesing, K. M., 1865. Revision der Protohelminthen. Abtheilung: Mastigophoren. *Sitzungsber. d. k. Akad. Wiss. zu Wien*, **52** (1): 287.

Ehrenberg, C. G. 1853. Ueber die neuerlich bei Berlin vorgekommenen neuen Formen des mikroskopischen Lebens. *Ber. Verh. d. k. preuss. Akad. Wiss. in Berlin*, **1853**: 183.

Kent, W. S. 1880–82. A Manual of the Infusoria, vols. **1**–**3**. London.

Klebs, G., 1883. Über die Organisation einiger Flagellatengruppen. *Unters. a. d. bot. Inst. Tübingen*, vol. **1**.

Klebs, G., 1892. Flagellatenstudien. *Zeitschr. f. wiss. Zool.*, **55**: 353.

Lauterborn, R., 1899. Protozoen-studien. IV. Theil. Flagellaten aus dem Gebiete des Oberrheins. *Zeitschr. f. wiss. Zool.*, **65**: 369.

Lemmermann, E., 1910. Kryptogamenflora der Mark Brandenburg, vol. **3**. Algen. Leipzig.

Levander, K. M., 1894. Materialien zur Kenntniss der Wasserfauna in der Umgebung von Helsingfors, mit besonderer Berücksichtigung der Meeresfauna. I. Protozoa. *Acta Soc. pro Fauna et Flora Fennica*, **12** (2): 1.

Lindau, G., and H. Melchior, 1926. Die Algen 1. Abt. 2. Aufl. In Lindau and Pilger, *Kryptogamenfl. f. Anfänger*, vol. 4 (1).

Maneval, W. E. Lactophenol preparations. To be published.

Mitrophanow, P., 1905. Étude sur la structure, le développement et l'explosion des trichocystes des Paramécies. *Arch. f. Protist.* **5**: 78.

Nowopokrowsky, J., 1911. Über die Chlorzinkjod-Reaktion der Zellulose. *Beih. z. bot. Centralbl.*, **28** (1): 90.

Oltmanns, F., 1922. *Morphologie und Biologie der Algen.* 2. Aufl. 1. Jena.

Pascher, A., 1913. *Die Susswasserflora Deutschlands, Österreichs und der Schweiz* **2**. Flagellatae II. Jena.

Scherffel, A., 1912. Zwei neue, trichocystenartige Bildungen führende Flagellaten. *Arch. f. Protist.* **27**: 94.

SCHUBERG, A., 1905. Über Cilien und Trichocysten einiger Infusorien. *Arch. f. Protist.* **6**: 61.

SENN, G., 1900. Flagellata. In Engler and Prantl, "Die Natürl. Pflanzenfam." **1** (1a): 93.

STEIN, F., 1878. Der Organismus der Infusionsthiere vol. 3 (1). Leipzig.

STOKES, A. C., 1886. Notices of New Fresh-water Infusoria. *Proc. Am. Phil. Soc.*, **23**: 562.

STOKES, A. C., 1888. A Preliminary Contribution toward a History of the Fresh-water Infusoria of the United States. *Jour. Trenton Nat. Hist. Soc.*, **1** (3): 71.

TURNER, C. L., 1917. A Culture Medium for Euglena with Notes on the Behavior of Euglena. *Anat. Rec.*, **12**: 407.

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COMPARISON OF THE REACTIONS AGAINST HETEROTRANSPLANTED TISSUES IN DIFFERENT KINDS OF HOSTS [1]

LEO LOEB

(From the Department of Pathology, Washington University School of Medicine, St. Louis, Missouri)

In several preceding papers, we have studied the reaction of organisms against heterotransplanted tissues, in particular as to the correspondence between these reactions and the phylogenetic relationships between host and transplants.[2] On the whole, we found a lack of such a correspondence, if we used mammalian and avian organisms as hosts, although it is present in more primitive classes of animals. However, even in mammalian hosts or transplants there seemed to be found at least an indication of such a correspondence; but this was not definite. We wished therefore to investigate this question again; comparing the behavior of two very nearly related species, such as rat and mouse, with more distant species, such as guinea pig and rat, cat and rat, pigeons and rat. We also compared in these investigations again some reciprocal transplantations, in order to determine on what factors the difference in the results depended; and lastly, we wished to study once more some of those modes of reactions which distinguished heterotransplantation from homoiotransplantation.

From a different point of view, namely, in their relation to strain or race transplants, we have considered already certain general aspects of transplantations from mouse to rat and the reciprocal transplantations.[3] We shall now consider these transplantations in their relation to heterotransplantations between further distant species, and we shall also compare the action of various tissues and cells of the hosts on these transplants. As usual, all our transplants were inserted into pockets in the subcutaneous tissue of the abdominal wall, and they were examined at different periods following transplantation. We

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[1] These investigations were carried out with the aid of a grant for research in science made to Washington University by the Rockefeller Foundation.

[2] Loeb, Leo, and W. H. F. Addison. *Arch. f. Entw.-mech.*, **27**: 73 (1909) and **32**: 44 (1911).

Loeb, Leo. *Jour. Exper. Med.*, **31**: 765 (1920); *Jour. Med. Res.*, **42**: 137 (1920–21).

Loeb, Leo, and J. S. Harter, *Am. Jour. Path.*, **2**: 521 (1926).

[3] Loeb, Leo and Helen D. King. *Am. Nat.*, **69**: 5 (1935).

used either thyroid and cartilage, together with the adjoining tissues, or cartilage with the adjoining tissues alone. In a few cases we grafted also the ovary. The transplants of thyroid and ovary were cut into complete serial sections and, in the case of the cartilage transplant, sections through the different parts of the transplant were examined. Altogether one hundred and thirty-four pieces were studied in this manner. We shall cite summaries of our findings at eight to ten and at twenty to thirty days following transplantations.

Eight to Ten Days after Transplantation

From rat to mouse and reciprocal transplantations.—In the rat to mouse transplants of thyroid, the grafted tissue has become necrotic; only in one specimen (after 8 days) were there still some acini with colloid preserved. Also muscle tissue transplanted with thyroid was necrotic. Fat tissue was largely necrotic and much infiltrated with connective tissue; a great many polymorphonuclear leucocytes were found especially in the necrotic areas. The surrounding connective tissue capsule was infiltrated with lymphocytes.

In the mouse to rat transplants of thyroid, the graft has been replaced by fibrous tissue, which is densely infiltrated with lymphocytes. In one specimen, there were some indistinct remnants of thyroid tissue seen. The transplanted ovaries were necrotic and, in the periphery, invaded by connective tissue, which transformed the transplant into a loosely vacuolar tissue. There was also noticeable some infiltration with lymphocytes.

In the rat to mouse transplants of cartilage, the cartilage and perichondrium were, in some cases, mostly preserved; in other cases, necrotic. Intermediate conditions were also found. The extent of infiltration of the transplant with polymorphonuclear leucocytes varied in different specimens; they were found especially in the necrotic fat tissue. There was also some definite lymphocytic infiltration, but it was not so marked as at later periods. The lymphocytes were found mostly in the distant connective tissue surrounding the transplants. The fat tissue was largely necrotic, especially near necrotic perichondrium.

In the mouse to rat transplants of cartilage, there was, on the average, a better preservation than in the reciprocal transplants. The perichondrium was partly replaced by lymphocytes, which penetrated a little into cartilage. Cartilage and perichondrium were surrounded by fibrous tissue densely infiltrated with lymphocytes. Necrotic cartilage was also invaded by connective tissue cells. The transplanted muscle tissue was necrotic. Bone and bone marrow were necrotic and the bone marrow was partly or completely replaced by connective tissue; there was a dense lymphocytic infiltration around the bone. After eight days, there was some infiltration with polymorphonuclear leucocytes, but after ten days, these cells had disappeared. In the fat tissue some hyperemic and hemorrhagic areas were found. In no case were there any regenerative processes seen in cartilage and muscle tissue. In specimens examined at six days transplanted cartilage and fat tissue were still preserved, as were acini of the thyroid at this time. There were large hemorrhagic areas in fat tissue. Again there was more fibrous tissue formation and lymphocytic infiltration in the mouse to rat than in the reciprocal transplantations. While there was here some infiltration with polymorphonuclear leucocytes, the maximum was reached after eight days.

From rat to guinea pig and reciprocal transplantations.—The results in the transplantations of thyroid from rat to guinea pig were very similar to those obtained after transplantation of this tissue from rat to mouse. In general, we found the transplanted acini as necrotic material in the center of the transplant. There was much infiltration with polymorphonuclear leucocytes in the dead tissue after eight days. In the peripheral tissue we found very moderate lymphocytic infiltration. In one specimen after eight days, there were, in the periphery of the transplant, some isolated acini with solid colloid in the connective tissue and in another specimen the outer epithelial walls of a few acini were preserved. Also after ten days, we found in one specimen a few peripheral parts of the walls preserved. The necrotic thyroid and fat tissue were still surrounded by a mantle of polymorphonuclear leucocytes, but in the surrounding connective tissue there was now more lymphocytic infiltration.

In the reciprocal transplants from guinea pig to rat there was, after eight days, some necrotic and hemorrhagic material being organized by fibroblasts and partly in process of hyalinization. There was a certain amount of diffuse lymphocytic infiltration, which was rather dense in some areas of connective and fat tissue. The infiltration with polymorphonuclear leucocytes was less in this case than in the reciprocal transplants. Especially in the ten-day transplants, the amount of necrotic thyroid tissue and the number of polymorphonuclear leucocytes were less than in the reciprocal transplants; but there was more lymphocytic infiltration and there was also a greater activity of phagocytes than in the reciprocal transplants.

In the eight-day cartilage transplants, we find some variations as to preservation of cartilage in different specimens, the preservation being, on the whole, greater in the guinea pig to rat than in the reciprocal transplants. Polymorphonuclear leucocytes moved into the necrotic fat and also into the necrotic cartilage tissue There were some remnants of hemorrhage in the fat tissue; there were also lymphocytes and connective tissue in the interstices between fat cells. Muscle tissue and bone marrow were necrotic or the necrotic tissue was replaced by fibrillar connective tissue. In the guinea pig to rat transplants the fat tissue was mostly replaced by fibrous tissue. Here, as in the mouse to rat transplants, the lymphocytes were more active; they infiltrated the connective tissue around bone and cartilage and penetrated a little way into the perichondrium. Again, similarly to the mouse to rat transplants, there was here more fibrous tissue formation, a more marked infiltration with lymphocytes, and less infiltration with polymorphonuclear leucocytes. After ten days, more connective tissue and lymphocytes and fewer polymorphonuclear leucocytes penetrated into the necrotic cartilage and bone than in the rat to guinea pig transplants. Lymphocytes moved also into cartilage and bone which they helped to destroy and these cells could replace fat tissue. As in the mouse to rat transplants, there was more connective tissue and lymphocytic activity and less necrosis in the guinea pig to rat than in the reciprocal transplants.

Transplantation of cartilage from cat to rat.—Cartilage could be preserved. Necrotic muscle and bone were replaced by connective tissue. Fat tissue was partly necrotic, partly replaced by connective tissue; the infiltration with cells of a different nature made it difficult to determine whether some fat cells were still alive. Much dense lymphocytic infiltration was found in surrounding tissue. In these transplants polymorphonuclear leucocytes were not as prominent as in the rat to mouse transplants. The lymphocytic infiltration around cartilage and in muscle was less marked than in transplants from mouse to rat. No regeneration was observed in perichondrium or muscle tissue.

Transplantation of cartilage from rat to pigeon.—Cartilage and perichondrium were either necrotic or parts of it, varying in amount in different cases, were preserved. The other transplanted tissues, including the fat tissue, were necrotic. Hemorrhages were found in the fat tissue and the latter was invaded by polymorphonuclear leucocytes, which moved also into the necrotic cartilage and muscle tissue. There were some phagocytic cells between the fat cells. Lymphocytic infiltration was still slight at this time.

Twenty to Thirty Days after Transplantation

Mouse to rat and reciprocal transplantations.—The transplanted thyroid had in all cases been replaced by fibrous tissue with lymphocytic infiltration; but in the mouse to rat transplantations, the lymphocytic infiltration was, as usually, much denser than in the reciprocal transplantations. In the rat to mouse transplants, on the other hand, a few polymorphonuclear leucocytes were still seen. The transplanted pieces of ovary were necrotic and surrounded by lymphocytes. Cartilage and perichondrium were entirely necrotic in the rat to mouse transplantations, but in about one-half of the specimens transplanted from mouse to rat the greater part of these tissues were preserved, while the other pieces were entirely necrotic. In both transplants lymphocytes penetrated into, destroyed, and partially replaced necrotic cartilage and perichondrium; also connective tissue penetrated into necrotic cartilage. On the other hand, these cells did not seem to invade preserved transplanted cartilage. The transplanted fat tissue was either replaced by masses of lymphocytes or connective tissue and when the fat cells were still preserved, lymphocytes, connective tissue, and vacuolated phagocytic cells had penetrated into the interstices between the fat cells and separated them. Transplanted muscle tissue, bone, and bone marrow were necrotic; bone marrow had been replaced by connective tissue and lymphocytes. Connective tissue grew also into the dead bone, around which giant cells had formed. The formation of a fibrous capsule, as well as lymphocytic infiltration, was more marked in the mouse to rat than in the reciprocal transplants; this accords with what we found also in earlier stages. Especially the lymphocytic infiltration was more dense in the mouse to rat transplants; this is true too of the connective tissue around necrotic cartilage and bone. But also in the rat to mouse transplants there was occasionally a more marked lymphocytic infiltration around the cartilage. Polymorphonuclear leucocytes were found in the connective tissue surrounding necrotic fat tissue and they also penetrate into the latter; but these cells were not frequent at this period and they were especially rare in the mouse to rat transplants. We see then that between twenty and thirty days following transplantation almost all the tissues are necrotic or replaced by connective tissue and lymphocytes.

Guinea pig to rat and reciprocal transplantations.—After twenty days we still found necrotic material in the center of the thyroid transplant; it was surrounded by a connective tissue capsule which was much infiltrated with lymphocytes; there were among the latter also some polymorphonuclear leucocytes. Connective tissue cells slowly invaded and organized the central necrotic material. In these transplants, the invading connective tissue cells first converted the necrotic material into a hyaline fibrillar material; this represented the first step in the organization. Cells with much cytoplasm then moved with pseudopods into the hyaline material; they enclosed particles of the latter in their cell body and digested them. Thus a vacuolated tissue developed. After twenty-five days the necrotic material had disappeared and only nodules of fibrillar connective tissue were left. These were infiltrated with lymphocytes. Some polymorphonuclear leucocytes were still present in certain nodules, probably left over from the former stage, when there was still necrotic material present.

Transplantation of cartilage.—At twenty days, cartilage and perichondrium were mostly necrotic; small parts were preserved in the rat to guinea pig transplants and somewhat more in the reciprocal transplants. Fat tissue and bone marrow were necrotic or replaced by fibrillar connective tissue; muscle tissue was necrotic. In the rat to guinea pig transplants some polymorphonuclear leucocytes moved into the necrotic cartilage and fat tissue. Lymphocytes infiltrated the fat tissue. In the guinea pig to rat transplants lymphocytic infiltration was found around the necrotic bone, connective tissue, and cartilage; lymphocytes penetrated also into the perichondrium. In these transplants there was more fibrous tissue formation, more lymphocytic infiltration, and less invasion by polymorphonuclear leucocytes than in the reciprocal transplants. After twenty-five days, the transplanted tissues were necrotic, except in one piece, in which in the guinea pig to rat transplant, cartilage cells were in some parts still preserved. Connective tissue cells with many lymphocytes invaded the necrotic cartilage. There were also many polymorphonuclear leucocytes, in certain cases, around the necrotic cartilage. Fat tissue was either replaced by fibrillar connective tissue or the septa between the fat cells were invaded by connective tissue cells and by cells which took up small fat droplets. In the connective tissue, which had replaced part of the fat tissue, polymorphonuclear leucocytes could be seen in some instances. In the necrotic fat tissue giant cells were found and giant cells formed also around necrotic cartilage and muscle tissue.

In transplants of cartilage from cat to rat after twenty days.—Parts of cartilage and perichondrium were preserved; other parts were necrotic. Around the cartilage the connective tissue was densely infiltrated with lymphocytes and from here lymphocytes invaded the cartilage and dissolved it. Fat tissue was mostly necrotic. In the fibrillar connective tissue surrounding it there was much lymphocytic infiltration and these lymphocytes and connective tissue cells pushed their way between fat cells; again we found here vacuolar phagocytic cells which had taken up fat droplets. The muscle tissue was necrotic and in process of organization by connective tissue. In the connective tissue capsule surrounding the piece there was much lymphocytic infiltration. It may be stated—and this applies also to other cases in this series—that it is often difficult to determine whether or not small parts of the transplanted fat tissue are still alive, when so many cells coming from the host tissue push their way into the interstices between the fat cells.

Transplantation of cartilage from rat to pigeon; examination twenty and twenty-five days after transplantation.—After twenty-five days, the transplants were entirely necrotic, and giant cells were seen around the pieces. After twenty days, however, there were in certain areas of the cartilage some cells still preserved. In one place at the border of cartilage and perichondrium, the hyaline tissue was dissolved, owing to the taking up of fluid by this tissue, which thus bulged out. The cartilage cells were preserved and had cell processes in this soft material. In some specimens after twenty and twenty-five days strands of lymphocytes penetrated into the necrotic perichondrium or also into the cartilage and into interstices in the dense connective tissue surrounding the cartilage; with the lymphocytes, in some cases, polymorphonuclear leucocytes invaded the necrotic cartilage. The fat tissue was necrotic; it was either replaced by connective tissue or by a tissue consisting of vacuolated cells, which evidently had taken up fat droplets. Lymphocytes surrounded single and coalesced fat cells and furthermore collections of polymorphonuclear leucocytes were found in the fat tissue. The transplant was encircled by node-like collections of lymphocytes, between which a diffuse infiltration of lymphocytes was found in the small-celled, fibrillar connective tissue. Mitoses were occasionally seen in these lymphocytes. It was especially the necrotic material which attracted the polymorphonuclear leucocytes.

Discussion

If we consider this entire series of heterotransplantations of thyroid and cartilage with the surrounding tissues, we come to the conclusion that in all cases the destruction of the transplants has been almost complete at a relatively early date following transplantation. There were up to the tenth day still a few acini exceptionally preserved in the rat-mouse and in the rat-guinea pig transplantations, but not in other, more distant heterotransplantations. Parts of the transplanted cartilage were in some of the hosts still preserved up to twenty days, apparently without regard to relationship. Bone, bone marrow, fat tissue, muscle tissue, and with the few exceptions mentioned, thyroid and parathyroid tissue were always necrotic. In regard to the fat tissue we must make the reservation that when this is densely infiltrated with various kinds of cells, it may be very difficult to exclude the possibility that some fat cells may not still be preserved; but in no case could this be definitely established. Even if some cells were preserved, as we found so often in the case of cartilage cells, it is very improbable that these morphological criteria of life implied that the function and metabolism of these cells were normal. We certainly did not observe eight days and later, following transplantation, any growth processes in any of the transplanted tissues, in contrast to what we observe after homoio- and strain transplantation, where regenerative processes in certain tissues are very common. In periods earlier than eight days, we found a few doubtful cases of possible slight proliferative processes in mouse-rat transplants.

It is then this very pronounced necrosis of tissues after heterotransplantation, which evidently makes impossible a definite correspondence between the results of these transplantations, and the phylogenetic relationship between host and transplant. Indications of such a correspondence are perhaps present. Thus we find in the transplantations of cartilage and surrounding tissues from rat to pigeon, on the whole, a more marked infiltration with polymorphonuclear leucocytes than in other kinds of transplantations; but after all the interpretation of such differences is doubtful.

As to the cause of this rapid destruction, there can be no doubt that after heterotransplantation, it is mainly the toxicity of the body fluids which injures tissues directly and which in particular is responsible for the lack of the establishment of a satisfactory connection between the vessels of the host and transplant. These conditions lead to early death of the heterogenous grafts. Contrary to our findings in many cases in homoio- and syngenesiotransplantation, the injurious action of the lymphocytes and connective tissue of the host cannot be held responsible for the death of the transplants. There may be much activity of the connective tissue; but it consists mainly in the invasion of dead tissue and its gradual organization. Also dense collections of lymphocytes are often found; but lymphocytes usually infiltrate the connective tissue, replacing and surrounding the transplanted tissues. They may furthermore infiltrate the interstices of the necrotic fat tissue and even the perichondrium, cartilage, and bone; but all these tissues are by this time either dead or at least very much weakened in their vitality by the injurious conditions of the body fluids of the host; they are not primarily destroyed by lymphocytes. However, as stated, lymphocytes often are present in very large numbers in the fibrous tissue surrounding and replacing the heterogenous grafts.

In our earlier papers on heterotransplantation, we noticed the characteristic appearance of polymorphonuclear leucocytes which contrasted with their usual absence in homoio- and syngenesiotransplantations. In these experiments, we confirmed our previous observations in this respect, but we analysed still further the occurrence of these cells and found it very probable that they are not attracted by the heterotransplant as such, but by the heterogenous tissue when it has undergone necrosis, especially the necrotic fat and thyroid tissue. They usually disappear with the progressive organization of the necrotic tissue, but may still be found in fibrous tissue which has replaced the necrotic material, at a stage when the latter has completely or almost completely disappeared. We may assume that under those conditions they were remnants of the larger number of leucocytes, which had been attracted by the necrotic material at a previous stage. In certain heterotransplants, polymorphonuclear leucocytes may be very significant or altogether lacking; thus they were very inconspicuous after transplantation of cartilage of the cat into the rat. On the other hand, they were often present in very large collections around transplantations of cartilage and adjoining pieces from rat into pigeon. If it is mainly the necrotic material which attracts the polymorphonuclear leucocytes after heterotransplantation, then we must inquire why they are not attracted by the necrotic material which we find after homoiotransplantation, where we usually observed them only in the first few days after transplantation around the grafted tissue, after which period they disappear. We must assume that the autolytic processes, which take place in the necrotic tissue, are not exactly the same in these two types of transplantation: or we may consider the difference in vascularization around and in homoiogenous grafts as the important factor. It seems that the organization of the necrotic material takes place more rapidly after homoiotransplantation than after heterotransplantation. In the past we considered also the possibility that a difference in a possible contamination with microorganisms might be responsible for the presence or absence of these cells. However, the technique used by us in heterotransplantation is the same as that employed in the case of homoiotransplantation, and in the latter, as stated, these accumulations of polymorphonuclear leucocytes are usually absent. Moreover, in special experiments made from this point of view, we did not observe that bacteria, adherent to heterotransplanted epidermis and hair, behaved differently from bacteria adherent to similar material after homoiotransplantation.

We may also conclude that no very distinct differences are found between heterotransplantations which differ in respect to the nearness or distance of relationship between donor and host, a result that can be readily understood, if we consider the very great intensity of the injury inflicted upon the grafts by heterotransplantations between even nearly related species, such as mouse and rat. However, there are some indications that, also in heterotransplantations, relationship still plays a certain rôle in the results; thus transplantation of pigeon skin to chicken is more favorable than transplantation of pigeon skin to mammals. Furthermore, transplantations of pigeon skin to frogs lead to a rapid destruction of the graft.

In addition, certain differences do exist between the reactions in different species, which function as hosts for the various transplants. Thus it appears that in the mouse and in the guinea pig, the reaction on the part of the polymorphonuclear leucocytes is more intense than in the rat, and there are also indications that, on the contrary, the organizing activity of the connective tissue and the invasion by lymphocytes is stronger in the rat than in the mouse and guinea pig. In the pigeon we found the peculiarity that, in the earlier stages following transplantation, the invasion of necrotic material by polymorphonuclear leucocytes is very marked, while in later stages the accumulation of lymphocytes around the transplant predominates; as in cases of homoiotransplantation in birds, so also after heterotransplantation, follicle-like accumulations of lymphocytes develop in the circumference of the graft. These observations agree also with our former observations, which showed that in the guinea pig and rabbit the leucocytic reaction was more pronounced than in some other species and that therefore reciprocal transplantations may give different results. This was evident, for instance, in the transplantation of guinea pig kidney to pigeon and the reciprocal transplantation of pigeon kidney to guinea pig and also in the transplantation of this tissue to rat.

The outcome is different if we carry out heterotransplantations in the more primitive phylogenetic and ontogenetic stages. Then the reactions against the strange tissues are more moderate, the maximum of response has not yet been reached, when tissues are exchanged between related species, and there is therefore in these cases a more definite correspondence between phylogenetic relationship and the result of the transplantation. Furthermore, in the majority of transplantations in lower organisms we have to deal with a condition analogous to parabiosis, rather than to transplantation in the restricted sense.

Conclusion

1. After heterotransplantation, even between nearly related species, a relatively early necrosis of the grafted tissues occurs, the rapidity

of which varies, however, irrespective of their hosts, in accordance with the resistance of the tissues used.

2. Finer gradations between the results of transplantations in accordance with the phylogenetic relationship of host and transplant cannot be established, the necrosis of the grafted tissues being rapid even in nearly related species serving as hosts; but it is possible that, after heterotransplantations to still farther distant classes, such as amphibians, the mammalian or avian tissues undergo a more rapid and complete destruction than they do after transplantation to other mammalian or avian species.

3. In this destruction, body fluids play the principal part. In general, polymorphonuclear leucocytes appear in larger numbers and for a longer period of time after heterotransplantation, than after homoio- and interracial transplantations; but it is probable that these cells are attracted mainly by certain necrotic heterogenous tissues and they appear with special frequency in certain species. Also lymphocytes gradually appear in larger numbers, particularly in the fibrous tissue surrounding and organizing the transplants.

4. Various species, acting as hosts, differ in the intensity and in the kind of their reactions towards heterotransplants, irrespective of their relationship to the grafts. Reciprocal transplantations may therefore differ as to their results.

ON THE ENERGETICS OF DIFFERENTIATION

II. A COMPARISON OF THE RATES OF DEVELOPMENT OF GIANT AND OF NORMAL SEA-URCHIN EMBRYOS

ALBERT TYLER[1]

(*From the William G. Kerckhoff Laboratories of the Biological Sciences, California Institute of Technology, Pasadena, California*)

The following experiments show that normally proportioned giant embryos produced by the fusion of two fertilized eggs develop more rapidly than do normal embryos. This result had been anticipated (Tyler, 1933) on the basis of some experiments on the oxygen consumption and rate of development of half-sized embryos.

Theoretical Part

It has been shown (Tyler, 1933) that the rate of oxygen consumption of two half-sized embryos from isolated blastomeres is the same as that of one normal embryo up to the gastrula stage. The dwarf embryos, however, develop more slowly than the normal. Thus, when compared at the same stage of development (e.g., end of gastrulation), the total oxygen consumption of two dwarf embryos is greater than that of a single normal embryo. The theory upon which these results are interpreted involves the following assumptions: (1) Work is performed (and energy thus required) in the process of gastrulation as in other form changes in embryonic differentiation. (2) The amount of work performed (or energy required) varies directly with the amount of material involved and inversely with some function of the radius of curvature of the embryo or of the part considered. (3) The oxygen consumption is a measure of the energy liberated.

On the basis of assumptions 1 and 2, two dwarf embryos from isolated blastomeres should perform more work (and thus require more energy) for the process of gastrulation than one normal embryo. The experiments show that two dwarf embryos have the same rate of oxygen consumption as one normal embryo. On the basis of assumption 3 this means that energy is liberated at the same rate in the two dwarf embryos as in the normal embryo. The slower development of the dwarf embryo was thus accounted for, since with half the amount of energy available per unit time and with more than half the amount of work to be performed, more time is required to supply the amount of energy necessary for the various processes.

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[1] National Research Council Fellow in the Biological Sciences at the Zoölogical Station in Naples during the early part of this work.

These considerations led to the expectation that giant embryos produced by the fusion of two fertilized eggs should develop faster than the normal embryos.

Experimental Part

Methods of fusing eggs have been described by Morgan (1895), Driesch (1900), Bierens de Haan (1913), Goldfarb (1913, 1915), von Ubisch (1925), Hörstadius (1928) and Balinsky (1932) for sea urchins and by Mangold (1920) and Mangold and Seidel (1927) for salamanders.

Morgan simply removed the membrane shortly after fertilization and found that in a concentrated mass some eggs would fuse in pairs. Driesch treated the eggs with Ca-free sea water after removal of the fertilization membrane and also tried alkaline sea water and centrifuging. Bierens de Haan used essentially the same method. Goldfarb placed eggs in an isotonic or hypotonic solution of NaCl in sea water for considerable lengths of time. Von Ubisch allowed the eggs to remain in Ca-free sea water from the two to four-cell stage, and then brought them close together in ordinary sea water. Hörstadius fused groups of blastomeres with other groups of blastomeres or with whole eggs by bringing them together in a groove in a piece of cinema film in a dish of Ca-free sea water. Balinsky used the same method for fusing two whole eggs in the four-cell stage.

Most of the various methods of fusion were tried. In all the experiments the membranes are first removed either by shaking the eggs in a tube or sucking them up in a pipette one to three minutes after fertilization. If the eggs are fairly concentrated they tend to stick together after removal of the membrane in clumps of various numbers, as noted by Morgan (1895). The eggs are apparently capable of sticking to one another during the period of ten to fifteen minutes after fertilization in which time the ectoplasmic layer forms. If the eggs are packed together by centrifuging after the ectoplasm is formed they do not stick together very readily. Eggs that were fused in pairs either spontaneously or by centrifuging after removal of the membranes were followed in about 300 cases. In most of these, double blastulæ were formed which later separated, giving two normal embryos. In some, double gastrulæ were produced. One normal giant was obtained.

In another way (essentially that of Driesch) the eggs were made to stick together by placing them for two to five minutes in Ca-free sea water at various times after the formation of the ectoplasmic layer and then packing them together by gently centrifuging immediately after return to ordinary sea water. By this method three normal giants were obtained. The Ca-free sea water treatment and subsequent centrifuging was also applied to eggs in the two-, four- and eight-cell stages. Two normal giants were obtained from eggs so treated in the two-cell stage and 2 more from eggs treated in the four-cell stage out of more than 200 fused eggs that were isolated.

Eggs were handled individually on cinema film according to the method of Hörstadius. Two normal giants were obtained; one from a pair of eggs fused in the two-cell stage and another from a pair fused in the four-cell stage.

The eggs were also handled individually and fused in capillary tubes. A number of capillary tubes sealed at one end are prepared (Fig. 1). The sealed end should have a rounded bottom obtained by blowing as the end is sealed off. About ¾ inch is a convenient length of tube. The tubes are first filled with Ca-free sea water by driving them down in a centrifuge tube containing that solution. They are then mounted vertically in a vial in a Stender dish containing Ca-free sea water so that their open ends are immersed. The eggs are first washed in Ca-free sea water and then, immediately, two eggs are placed in each capillary tube. Into some tubes only a single egg is inserted to serve as a control. The tubes are then transferred to a centrifuge tube containing ordinary sea water and are run at about 2,000 r.p.m. for 15 seconds to 3 minutes. The eggs are thus driven to the bottom of the capillary tube where they may fuse if the tube

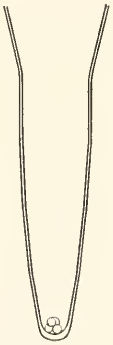

FIG. 1. Capillary tube for fusion of eggs.

is of the proper size and shape. During this time the ordinary sea water gradually diffuses into the capillary tube. If the eggs are allowed to remain in the tube they may develop into swimming blastulæ. It is preferable, however, to remove them at an early stage by

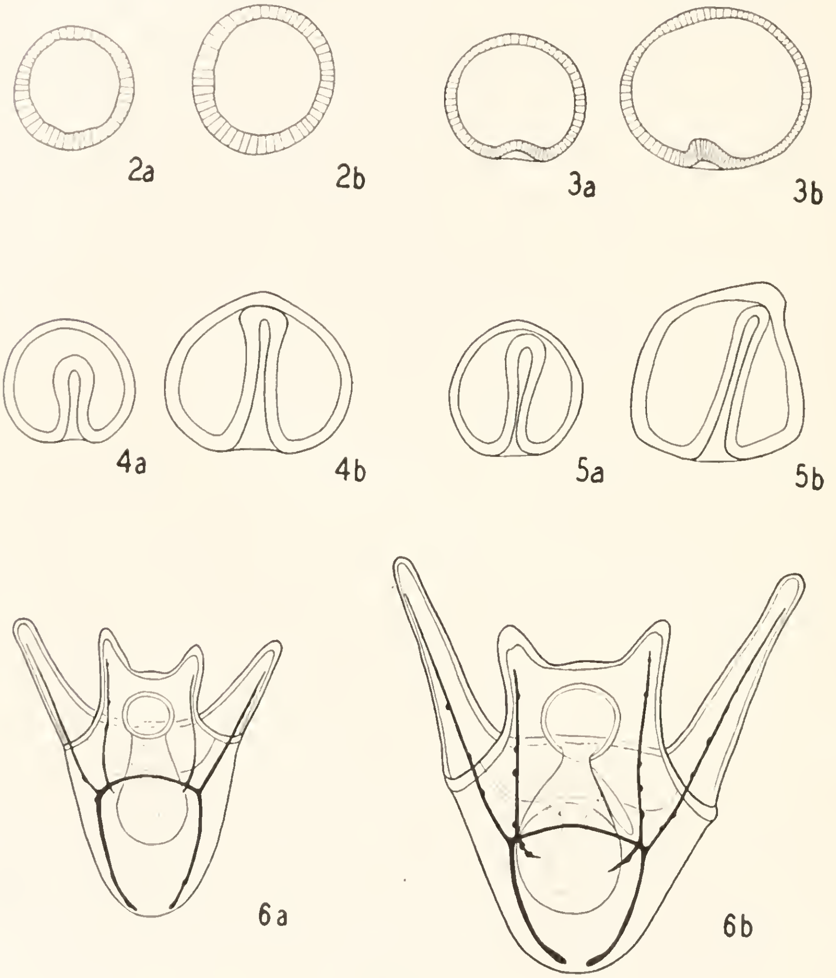

PLATE I

Giants from fused eggs and normal embryos of *Lytechinus*, drawn from photographs of living embryos.

FIGS. 2*a* and *b*. Normal and giant embryos in blastula stage.
FIGS. 3*a* and *b*. Normal and giant embryos in beginning gastrula stage.
FIGS. 4*a* and *b*. Normal embryo about two-thirds gastrulated, giant embryo completely gastrulated.
FIGS. 5*a* and *b*. Normal embryo completely gastrulated, giant embryo in prism stage.
FIGS. 6*a* and *b*. Normal and giant embryo in pluteus stage.

breaking off the sealed end and inverting it in a dish of sea water. The eggs will then slowly drop out. Four normal giants were obtained from more than 150 fused eggs that were followed. Two of them were from eggs fused in the one-celled stage and 2 from eggs fused in the two-cell stage.

A total of 14 normal giants were thus obtained. Of these 10 developed faster than the normal control eggs and 4 developed at the same rate as the controls. The results are presented in Table I. The sea urchins used in these experiments were *Sphærechinus granularis* and *Echinus microtuberculatus* at Naples and *Strongylocentrotus purpuratus* and *Lytechinus* at Corona del Mar, California. The material used and the method of treatment seemed to have no particular relation to the results.

The temperature was not controlled but single eggs treated in the same manner as the fused eggs were isolated in the same dish with the latter. These control eggs are listed with the giant eggs in the table. In four cases (Nos. 4, 7, 8, and 12) the giant and control embryos were found to be developing at the same rate. In the others the giants showed a faster rate of development. The difference in the rate of development is seen at the end of gastrulation and becomes more marked in the later stages. Thus in No. 11 the giant embryo is in the prism stage at a time when the control is still in the gastrula stage; in Nos. 6, 11, and 13 the giants are in the beginning pluteus stage while the corresponding controls are in the prism stage; and in Nos. 1, 3, 6, 11, 13, and 14 the giants are in the pluteus stage while the corresponding controls are entering that stage.

The giant embryos followed in these experiments were those that appeared normal throughout development. Those that showed two invaginations were discarded although they might sometimes give normal plutei according to Bierens de Haan (1913*c*) and Goldfarb (1917). Figures 2 to 6 show some of the giant embryos obtained. In Fig. 4*b* the giant embryo is a finished gastrula while the control embryo shown in Fig. 4*a* is about two-thirds gastrulated. Another giant embryo (Fig. 5*b*) is in the prism stage while the control embryo (Fig. 5*a*) is in the gastrula stage. In Figs. 6*a* and *b* a normal and a giant pluteus at 3½ days are shown. The giant embryo entered the pluteus stage at 64 hours after fertilization and the normal at 82 hours.

The evidence shows then that the giant embryos develop more rapidly than the normal. They complete gastrulation sooner than the controls and the difference in rate of development increases as development continues. The data is not extensive enough to decide whether they both begin gastrulation at the same time, a point that would be of some interest to determine.

TABLE I

The time (in hours) at which various developmental stages of the giant and normal embryos are attained.

	Beginning Gastrula	Completed Gastrula	Prism Stage	Beginning Pluteus	Pluteus
1. *Sphærechinus:*					
Giant	—	22	—	—	50
Control	—	25	—	50	62
2. *Echinus:*					
Giant	—	23	—	41	—
Control	—	25	41	—	—
3. ———					
Giant	16	19	—	50	58
Control	16	21	—	58	65
4. ———					
Giant	—	25	—	—	—
Control	—	25	—	—	—
5. *Strongylocentrotus:*					
Giant	26	30	—	—	—
Control	25	34	—	—	—
6. ———					
Giant	21	26	—	55	66
Control	21	29	55	66	79
7. *Lytechinus:*					
Giant	—	24	—	—	75
Control	—	24	—	—	75
8. ———					
Giant	19	24	—	—	—
Control	19	24	—	—	—
9. ———					
Giant	23	28	—	—	—
Control	25	32	—	—	—
10. ———					
Giant	25	30	—	—	—
Control	25	33	—	—	—
11. ———					
Giant	—	—	34	44	60
Control	—	34	44	60	—
12. ———					
Giant	—	30	41	—	—
Control	—	28	41	—	—
13. ———					
Giant	21	26	35	45	64
Control	22	30	45	64	82
14. ———					
Giant	—	28	39	—	65
Control	—	29	41	65	80

Discussion

The more rapid development of the giant embryos from fused eggs may be interpreted on the same basis as the slower development of the dwarf embryos from isolated blastomeres; namely, that twice as much energy is available per unit time in the giant embryo, but less than twice the amount of energy is required for gastrulation and other form changes. The giant embryos thus proceed faster in development than the normal embryos.

The slower development of the half-sized embryos has been noted by Morgan (1895, 1903), Driesch (1900, 1908), Hörstadius (1928) and Tyler (1933) for the case of the sea urchin; by Morgan (1901) and Driesch (1903) in *Amphioxus;* by Spemann and Falkenberg (1919) and Fankhauser (1930) in *Triton;* by Tyler (1930) in *Chætopterus.*

To interpret this slower development it has been assumed that a regulation process (whatever that might be) is involved in the formation of a whole embryo from an isolated blastomere and that the regulation requires time. Also to account for the still slower development of the embryos from ¼-blastomeres (Driesch, 1900), it must be assumed that they require even more time for regulation. But when a single embryo is produced from two eggs there certainly must be as much regulation involved as when two embryos are produced from a single egg. On that basis we should expect the giant embryos to develop slower than the normal. Driesch (1900), however, reported that the giant embryo develops as fast as the normal, and the present results show that an even faster rate of development is obtained. Another argument against the assumption that a time interval for regulation accounts for the slower development of the dwarf embryos was presented by Spemann and Falkenberg (1919). They found that in an unequally constricted egg the smaller fragment develops slower than the larger, both being slower than the whole egg. The difference was found to increase during development and was greater than the amount of time that they would care to assume for regulation. They concluded that the factors causing the slower development must be associated with the smaller size of the embryo.

The faster development of the giant embryos from fused eggs has a parallel in the faster development of giant limbs from fused limbbuds reported by Filatow (1932). Filatow grafted a limb-bud of one embryo on to that of another embryo in the same stage. The normal giant limb that developed was considerably further advanced in differentiation than the normal limb of the other side at the times of examination. Although this result in a general way fits in very well with the views expressed here, it is perhaps at present inadvisable to push the parallelism too far.

Another experiment that bears more directly on this work is that of Spemann and Bautzmann (1927). They produced giant and dwarf embryos by cutting two beginning gastrulæ of *Triton* parasagittally, one to the left, the other to the right of the mid-line, and fused the two larger pieces together as well as the two smaller pieces. In cases in which both fused embryos developed normally, the giant embryo developed markedly faster than the dwarf embryo. It is of interest to note that they say in this connection:—"Das gilt nun ganz allgemein und hängt nicht etwa mit irgendeiner grösseren Schädigung des kleineren Keimes zusammen oder mit einer grösseren Schwierigkeit, die er bei der Regulation zu überwinden gehabt hätte, sondern einfach mit seiner geringeren Grösse und den damit wohl gegebenen grösseren Widerständen bei all den Verlagerungen und Faltungen, mit denen die Entwicklung verbunden ist." This greater resistance in the displacements and foldings of development of which Spemann and Bautzmann speak is one of the main assumptions of the theory developed here (see p. 1) and presented in the earlier work (Tyler, 1933). It is essentially what is meant by the statement (Tyler, 1933, p. 156) that "when similar changes in shape are produced, more work is performed in the case of two half-embryos than in one whole embryo." It is interesting that Spemann and Bautzmann also find this a reasonable assumption to make. It would, however, be well to obtain further justification for this assumption, and a mathematical attempt to do this on purely mechanical considerations is now in progress.

The purpose of studying the energetics of development is to obtain information that would be of value in determining the causes of the form changes. Such information is of value for the problem of differentiation in the same way that a knowledge of the energetics of muscular contraction is of importance in determining the chemical reactions responsible for that process. In the case of differentiation, however, it has been contended that no energy is required for the form changes (Needham, 1932). This conclusion is drawn from the measurements of Meyerhof (1911) and of Shearer (1922), showing a constant ratio of heat production to oxygen consumption throughout development. It was assumed that if energy were required for the form changes then the heat evolved per mole of oxygen consumed should decrease at times when work was being performed. Among the objections that may be raised against this point of view it may be pointed out that heat bears no labels—the energy may have been very useful in producing the form changes before it appeared as heat. There is no more reason for expecting the energy of differentiation to be stored up in the embryo than that it should be stored up in plastic deformation of non-living objects. We conclude from the work on dwarf embryos (Tyler, 1933) and on the giant embryos that energy is required for the form changes. As was pointed out in the previous paper, the reason for the indirect method of attack is that we may recognize at least two other processes in development, namely maintenance and growth, which may require energy and which it was necessary to eliminate as factors in the result. The problem of obtaining a quantitative estimate of the energy required for differentiation may now be attacked and a number of methods suggest themselves. Among them the effect of temperature on the rate of development and on the rate of oxygen consumption is being investigated since if maintenance, growth, and differentiation should be found to have different temperature coefficients it would help in the analysis of the problem.

A question that has troubled embryologists for some time is why embryos from $^1\!/_8$-blastomere may develop to the gastrula stage and then stop. As a possible explanation of the failure to develop further, it may be suggested that the tiny embryos do not have enough energy left to go on, but expend it all on the relatively increased work of gastrulation and on the accompanying maintenance and growth requirements.

Summary

1. Giant embryos produced by the fusion of two fertilized eggs develop more rapidly than normal sized embryos.

2. The bearing of this result on the energetics of differentiation is discussed.

3. A new method of fusing eggs is described.

LITERATURE CITED

Balinsky, B., 1932. Interaction of Two Heteropolar Equipotential Systems Studied by the Method of Conplantation of Morulae of the Sea-urchin Strongylocentrotus droebachiensis. *Jour. du Cycle Bio-Zool. de l'Acad. des Sc. de l'Ukraine*, No. 1–2: 5.

Bierens de Haan, J. A., 1913*a*. Über bivalente Eier von Sphaerechinus granularis und die Grössenverhältnisse bei den aus diesen sich entwickelnden Larven. *Zool. Anz.*, **42**: 500.

Bierens de Haan, J. A., 1913*b*. Über homogene und heterogene Keimverschmelzungen bei Echiniden. *Arch. Entw.-mech.*, **36**: 474.

Bierens de Haan, J. A., 1913*c*. Über die Entwicklung heterogener Verschmelzungen bei Echiniden. *Arch. Entw.-mech.*, **37**: 420.

Driesch, H., 1900. Studien über das Regulationsvermögen der Organismen 4. Die Verschmelzung der Individualität bei Echinidenkeimen. *Arch. Entw.-mech.*, **10**: 411.

Driesch, H., 1903. Drei Aphorismen zur Entwicklungsphysiologie jüngster Stadien. *Arch. Entw.-mech.*, **17**: 41.

Driesch, H., 1908. Zur Theorie der organischen Symmetrie. *Arch. Entw.-mech.*, **26**: 130.

Driesch, H., 1910. Neue Versuche über die Entwicklung verschmolzener Echinidenkeime. *Arch. Entw.-mech.*, **30**: 8.

Fankhauser, G., 1930. Die Entwicklungspotenzen diploidkerniger Hälften des ungefurchten Tritoneies. *Arch. Entw.-mech.*, **122**: 671.

FILATOW, D., 1932. Entwicklungsbeschleunigung in Abhängigkeit von einer künstlichen Vergrösserung der Anlage. Versuche an Amphibienauge und -extremitaten. *Zool. Jahrb., Abt. Zool. und Phys.*, **51**: 589.

GOLDFARB, A. J., 1913. Studies in the Production of Grafted Embryos. *Biol. Bull.*, 24: 73.

GOLDFARB, A. J., 1915. Experimentally Fused Larvae of Echinoderms, with Special Reference to their Skeletons. Part 2. Arbacia punctulata. *Arch. Entw.-mech.*, **41**: 579.

GOLDFARB, A. J., 1917. The Symmetry of Grafted Eggs in Relation to Giant Larvae Formation in Arbacia punctulata. *Biol. Bull.*, **32**: 21.

HÖRSTADIUS, S., 1928. Über die Determination des Keimes bei Echinodermen. *Acta Zool.*, **9**: 1.

MANGOLD, O., 1920. Fragen der Regulation und Determination an umgeordneten Furchungsstadien und verschmolzenen Keimen von Triton. *Arch. Entw.-mech.*, **47**: 249.

MANGOLD, O., UND F. SEIDEL, 1927. Homoplastische und heteroplastische Verschmelzung ganzer Tritonkeime. *Arch. Entw-mech.*, **111**: 593.

MEYERHOF, O., 1911. Untersuchungen über die Wärmetönung der Vitalen Oxydationsvorgänge in Eiern. I, II, III. *Biochem. Zeitschr.*, **35**: 246.

MORGAN, T. H., 1895. Studies of the "Partial" Larvae of Sphaerechinus. *Arch. Entw.-mech.*, **2**: 81.

MORGAN, T. H., 1901. The Proportionate Development of Partial Embryos. *Arch. Entw.-mech.*, **13**: 416.

MORGAN, T. H., 1903. The Gastrulation of the Partial Embryos of Sphaerechinus. *Arch. Entw.-mech.*, **16**: 117.

NEEDHAM, J., 1932. Chemical Embryology, Cambridge.

SHEARER, C., 1922. On the Heat Production and Oxidation Processes of the Echinoderm Egg during Fertilisation and Early Development. *Proc. Roy. Soc.*, Ser. B, **93**: 410.

SPEMANN, H., UND E. BAUTZMANN GEB. WESSEL, 1927. Über Regulation von Tritonkeimen mit überschüssigem und fehlendem medianem Material. *Arch. Entw.-mech.*, **110**: 557.

SPEMANN, H., UND H. FALKENBERG, 1919. Über asymmetrische Entwicklung und Situs inversus viscerum bei Zwillingen und Doppelbildungen. *Arch. Entw.-mech.*, **45**: 371.

TYLER, A., 1930. Experimental Production of Double Embryos in Annelids and Mollusks. *Jour. Exper. Zool.*, **57**: 347.

TYLER, A., 1933. On the Energetics of Differentiation. A comparison of the oxygen consumption of "half" and whole embryos of the sea-urchin. *Pub. Staz. Zool. Napoli*, **13**: 155.

VON UBISCH, L., 1925. Entwicklungsphysiologische Studien an Seeigelkeimen. II. Die Entstehung von Einheitslarven aus verschmolzenen Keimen. *Zeitschr. wiss. Zool.*, **124**: 457.

INDEX

ALLEE, W. C. *See* Oesting and Allee, 314.

Antipolar lobe, of Ilyanassa, rhythmic changes in form, 296.

Antuitrin S, effects of extract on female lizard, Anolis carolinensis, 355.

Anura, pituitary-induced sexual reactions in, 74.

Aphids, wing-production, combinations of current and antecedent conditions in relation to, 35.

Arbacia punctulata, change in size and shape of ageing eggs, 180.

—— ——, viscosity changes in ageing unfertilized eggs, 191.

BACTERIA, lethal action of sunlight on, in sea water, 93.

Bacterial luminescence and cell integrity, 347.

BERKELEY, C. The chemical composition of the crystalline style and of the gastric shield; with some new observations on the occurrence of the style oxidase, 107.

BISSONNETTE, THOMAS HUME. Modification of mammalian sexual cycles, II, 300.

BROWER, HELEN PORTER. *See* Parker and Brower, 4.

BURGER, J. WENDELL, AND CHARLES STEAD THORNTON. A correlation between the food eggs of Fasciolaria tulipa and the apyrene spermatozoa of prosobranch molluscs, 253.

CALCIUM, protective effect on Proceredes, 314.

Cell integrity and bacterial luminescence, 347.

—— volume, aggregate, validity of centrifuge method for estimating, 363.

Centrifuge method, validity for estimating aggregate cell volume in suspension of the egg of the sea urchin, Arbacia punctulata, 347.

Centrifuging, effect on polarity of Griffithsia, 172.

——, eggs of Ilyanassa in reverse, 268.

——, separation of egg of Ilyanassa into two parts by, 280.

CHAO, IPING. Hydrogen-ion concentration and the rhythmic activity of the nerve cells in the ganglion of the Limulus heart, 69.

Chemical composition of crystalline style and gastric shield, 107.

Chromatophoral nerve-fibers in dogfish, electric stimulation, 1.

Chromosomes of Habrobracon, 25.

—— of Lepas anatifera, 263.

Ciliates associated with amphipod family Orchestiidæ from Woods Hole district, 51.

CLARKE, G. L., AND S. S. GELLIS. The nutrition of copepods in relation to the food-cycle of the sea, 231.

COHEN, AARON. *See* Drouet and Cohen, 422.

Color changes and isolated scale erythrophores of squirrelfish, 131.

Copepods, nutrition of, in relation to food-cycle of sea, 231.

Crayfish, Golgi bodies in nerve cells, 163.

Crystalline style, chemical composition of, 107.

Cupric chlorides, effect on cardiac explants in tissue culture, 215.

DAPHNIA, digestive enzymes of, 207.

DAWSON, ALDEN B. The hemopoietic response in the catfish, Ameiurus nebulosus, to chronic lead poisoning, 335.

Development, of sea urchin, action of lithium on, 378.

——, rates of, comparison of, in giant and normal sea-urchin embryos, 451.

Digestive enzymes of Daphnia, 207.

Distribution, vertical, of larger zoöplankton in deep water, 115.

Dogfish, electric stimulation of chromatophoral nerve-fibers, 1.

Doody, Thomas C. *See* Randall and Doody, 258.

Drouet, Francis, and Aaron Cohen. The morphology of Gonyostomum Semen from Woods Hole, Massachusetts, 422.

ECTOCOMMENSALS, associated with amphipod family Orchestiidæ from Woods Hole District, 51.

Egg, of Ilyanassa, centrifuging in reverse, 268.

—, — ——, separation into two parts by centrifuging, 280.

Eggs, ageing, of Arbacia punctulata, change in size and shape, 180.

Elliott, Alfred M. The influence of pantothenic acid on growth of Protozoa, 82.

Embryos, rates of development, in giant and normal sea urchins, 451.

Enzymes, digestive, of Daphnia, 207.

Erythrophores, isolated scale, and color changes of squirrelfish, 131.

Evans, Llewellyn Thomas. The effects of antuitrin S and sheep pituitary extract on the female lizard, Anolis carolinensis, 355.

FASCIOLARIA tulipa, food eggs and apyrene spermatozoa of prosobranch molluscs, 253.

Ferric chloride, effect on cardiac explants in tissue culture, 215.

Food-cycle of sea, nutrition of copepods in relation to, 231.

Food-eggs of Fasciolaria tulipa and apyrene spermatozoa of prosobranch molluscs, 253.

Fundulus heteroclitus, nuptial secondary sex character, 4.

GASTRIC shield, chemical composition, 107.

Gellis, S. S. *See* Clarke and Gellis, 231.

Goldforb, A. J. Change in size and shape of ageing eggs (Arbacia punctulata), 180.

——, ——. Viscosity changes in ageing unfertilized eggs of Arbacia punctulata, 191.

Golgi bodies in nerve cells of Cambarus, 163.

Gonyostomum Semen, morphology of, from Woods Hole, 422.

Growth of Protozoa, as affected by pantothenic acid, 82.

HABROBRACON, chromosomes of, 25.

Hasler, Arthur D. The physiology of digestion of plankton crustacea. I. Some digestive enzymes of Daphnia, 207.

Hemolytic action of photo-fluorescein, 360.

Hemopoietic response in the catfish, Ameiurus nebulosus, to chronic lead poisoning, 335.

Hermaphrodites, chromosomes of, 263.

Hetherington, Duncan C., and Mary E. Shipp. The effect of cupric, manganous and ferric chlorides upon cardiac explants in tissue culture, 215.

Hydrogen ion concentration and rhythmic activity nerve cells in ganglion Limulus heart, 69.

ILYANASSA, rhythmic changes in form of isolated antipolar lobe, 296.

——, separation of egg into two parts by centrifuging, 280.

JOHNSON, Martin W. The developmental stages of Labidocera, 397.

KIDDER, George W., and Francis M. Summers. Taxonomic and cytological studies on the ciliates associated with the amphipod family Orchestiidæ from the Woods Hole District. I. The stomatomous holotrichous ectocommensals, 51.

Kleinholz, L. H. The Golgi bodies in the nerve cells of the crayfish, Cambarus, 163.

Korr, Irvin M. Relation between cell integrity and bacterial luminescence, 347.

LABIDOCERA, developmental stages of, 397.

Lead poisoning, hemopoietic response in catfish, Ameiurus nebulosus, to, 335.

Leavitt, Benjamin B. A quantitative study of the vertical distribution of the larger zoöplankton in deep water, 115.

Lepas anatifera L., chromosomes of, 263.
Limulus, ganglion of heart, hydrogen ion concentration and rhythmic activity of nerve cells, 69.
Lithium, action on sea urchin development, 378.
Loeb, Leo. A comparison of the reactions against heterotransplanted tissues in different kinds of hosts, 440.
Luminescence, bacterial, and cell integrity, 347.
Lungs, of the manatee, 385.

MANGANOUS chloride, effect on cardiac explants in tissue culture, 215.
McEwen, George F. *See* Zobell and McEwen, 93.
Melanophores, deep-seated, responses of, in fishes and amphibians, 7.
Menke, John F. The hemolytic action of photo-fluorescein, 360.
Morgan, T. H. Centrifuging the eggs of Ilyanassa in reverse, 268.
——, — —. The rhythmic changes in form of the isolated antipolar lobe of Ilyanassa, 296.
——, — —. The separation of the egg of Ilyanassa into two parts by centrifuging, 280.

NERVE cells, Golgi bodies in, in crayfish, 163.
—— ——, hydrogen-ion concentration and rhythmic activity, in ganglion of Limulus heart, 69.
Nerve-fibers, chromatophoral, electric stimulation of, in dogfish, 1.
Nerves, living, studies of, 140.
Nuttycombe, John W., and Aubrey J. Waters. Stenostomum pseudoacetabulum (nov. spec.), 168.

OESTING, R. B., and W. C. Allee. Further analysis of the protective value of biologically conditioned fresh water for the marine turbellarian, Proceredes Wheatlandi. IV. The effect of calcium, 314.
Oxygen, low, of physiological salt solution, 258.

PANTOTHENIC acid, effect on growth of Protozoa, 82.
Parker, G. H. The electric stimulation of the chromatophoral nerve-fibers in the dogfish, 1.
——, — —, and Helen Porter Brower. A nuptial secondary sex character in Fundulus heteroclitus, 4.
Photo-fluorescein, hemolytic action of, 360.
Pigment cells, movement of, in eyes of crustaceans, evidence of diurnal rhythm, 247.
Pituitary-induced sexual reactions in Anura, 74.
Polarity, effect of centrifuging on, of Griffithsia bornetiana, 172.
Proceredes, protective value of biologically conditioned fresh water, 314.
Protozoa, growth of, influence of pantothenic acid on, 82.
Pyocyanine, influence on respiration sea urchin egg, 327.

RANDALL, Merle, and Thomas C. Doody. A buffered and low oxygen content physiological salt solution, 258.
Reaction against heterotransplanted tissues in different kinds of hosts, 440.
Respiration of sea urchin egg, influence of pyocyanine, 327.
Rhythm, diurnal, evidence of, in movement of pigment cells in eyes of crustaceans, 247.
Rhythmic changes in form of isolated antipolar lobe of Ilyanassa, 296.
Rugh, Roberts. Pituitary-induced sexual reactions in the Anura, 74.
Runnström, John. An analysis of the action of lithium on sea urchin development, 378.
——, ——. On the influence of pyocyanine on the respiration of the sea urchin egg, 327.

SALT solution, physiological, buffered and low oxygen content, 258.
Schechter, Victor. The effect of centrifuging on the polarity of an alga, Griffithsia bornetiana, 172.
Sears, Mary. Responses of deep-seated melanophores in fishes and amphibians, 7.
Sea urchin, development as affected by lithium, 378.

Sex character, nuptial secondary, in Fundulus, 4.
Sexual cycle, mammalian, modification of, 300.
—— reactions, pituitary-induced, in Anura, 74.
Shapiro, Herbert. The validity of the centrifuge method for estimating aggregate cell volume in suspension of the egg of the sea urchin, Arbacia punctulata, 363.
Sheep pituitary extract, effect on female lizard, Anolis carolinensis, 355.
Shipp, Mary E. *See* Hetherington and Shipp, 215.
Shull, A. Franklin. Combinations of current and antecedent conditions in relation to wing-production of aphids, 35.
Smith, Dietrich C., and Margaret T. Smith. Observations on the color changes and isolated scale erythrophores of the squirrelfish, Holocentrus ascensionis (Osbeck), 131.
Speidel, Carl Caskey. Studies of living nerves, 140.
Spermatozoa, apyrene, of prosobranch molluscs, and food eggs of Fasciolaria tulipa, 253.
Stenostomum pseudoacetabulum, 168.
Style oxidase, observations on, 107.
Summers, Francis M. *See* Kidder and Summers, 51.
Sunlight, lethal action on bacteria in sea water, 93.

Thornton, Charles Stead. *See* Burger and Thornton, 253.
Tissue culture, effect of cupric, manganous, and ferric chlorides upon cardiac explants, 215.
Tissues, heterotransplanted, reactions against, in different kinds of hosts, 440.
Torvik-Greb, Magnhild. The chromosomes of Habrobracon, 25.
Tyler, Albert. On the energetics of differentiation. II. A comparison of the rates of development of giant and of normal sea-urchin embryos, 451.

Viscosity changes in ageing unfertilized eggs of Arbacia punctulata, 191.

Water, biologically conditioned, protective value for marine turbellarian, 314.
Waters, Aubrey J. *See* Nuttycombe and Waters, 168.
Welsh, John H. Further evidence of a diurnal rhythm in the movement of pigment cells in eyes of crustaceans, 247.
Wing-production of aphids, combinations of current and antecedent conditions in relation to, 35.
Wislocki, George. Lungs of the manatee, 385.
Witschi, Emil. The chromosomes of hermaphrodites. I. Lepas anatifera L., 263.

Zobell, Claude E., and George F. McEwen. The lethal action of sunlight upon bacteria in sea water, 93.
Zoöplankton, vertical distribution of, in deep water, 115.

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CONTENTS

Page

PARKER, G. H.
The Electric Stimulation of the Chromatophoral Nerve-Fibers in the Dogfish . . . 1

PARKER, G. H., AND HELEN PORTER BROWER
A Nuptial Secondary Sex Character in Fundulus heteroclitus. 4

SEARS, MARY
Responses of Deep-seated Melanophores in Fishes and Amphibians . . . 7

TORVIK-GREB, MAGNHILD
The Chromosomes of Habrobracon . . . 25

SHULL, A. FRANKLIN
Combinations of Current and Antecedent Conditions in Relation to Wing-production of Aphids . . . 35

KIDDER, GEORGE W., AND FRANCIS M. SUMMERS
Taxonomic and Cytological Studies on the Ciliates Associated with the Amphipod Family Orchestiidae from the Woods Hole District. I. The stomatomous holotrichous ectocommensals . . . 51

CHAO, IPING
Hydrogen-ion Concentration and the Rhythmic Activity of the Nerve Cells in the Ganglion of the Limulus Heart . . . 69

RUGH, ROBERTS
Pituitary-Induced Sexual Reactions in the Anura . . . 74

ELLIOTT, ALFRED M.
The Influence of Pantothenic Acid on Growth of Protozoa . . 82

ZOBELL, CLAUDE E., AND GEORGE F. MCEWEN
The Lethal Action of Sunlight upon Bacteria in Sea Water . . 93

BERKELEY, C.
The Chemical Composition of the Crystalline Style and of the Gastric Shield; with Some New Observations on the Occurrence of the Style Oxidase . . . 107

LEAVITT, BENJAMIN B.
A Quantitative Study of the Vertical Distribution of the Larger Zoöplankton in Deep Water . . . 115

SMITH, DIETRICH C., AND MARGARET T. SMITH
Observations on the Color Changes and Isolated Scale Erythrophores of the Squirrelfish, Holocentrus ascensionis (Osbeck) . . . 131

SPEIDEL, CARL CASKEY
Studies of Living Nerves . . . 140

Volume LXVIII Number 2

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CONTENTS

Page

KLEINHOLZ, L. H.
The Golgi Bodies in the Nerve Cells of the Crayfish, Cambarus.... 163

NUTTYCOMBE, JOHN W., AND AUBREY J. WATERS
Stenostomum pseudoacetabulum (nov. spec.).................. 168

SCHECHTER, VICTOR
The Effect of Centrifuging on the Polarity of an Alga, Griffithsia bornetiana.. 172

GOLDFORB, A. J.
Change in Size and Shape of Ageing Eggs (Arbacia punctulata).. 180

GOLDFORB, A. J.
Viscosity Changes in Ageing Unfertilized Eggs of Arbacia punctulata 191

HASLER, ARTHUR D.
The Physiology of Digestion of Plankton Crustacea. I. Some digestive enzymes of Daphnia.................................. 207

HETHERINGTON, DUNCAN C., AND MARY E. SHIPP
The Effect of Cupric, Manganous and Ferric Chlorides upon Cardiac Explants in Tissue Culture.......................... 215

CLARKE, G. L., AND S. S. GELLIS
The Nutrition of Copepods in Relation to the Food-Cycle of the Sea.. 231

WELSH, JOHN H.
Further Evidence of a Diurnal Rhythm in the Movement of Pigment Cells in Eyes of Crustaceans....................... 247

BURGER, J. WENDELL, AND CHARLES STEAD THORNTON
A Correlation between the Food Eggs of Fasciolaria tulipa and the Apyrene Spermatozoa of Prosobranch Molluscs................. 253

RANDALL, MERLE, AND THOMAS C. DOODY
A Buffered and Low Oxygen Content Physiological Salt Solution. 258

WITSCHI, EMIL
The Chromosomes of Hermaphrodites. I. Lepas anatifera L.... 263

MORGAN, T. H.
Centrifuging the Eggs of Ilyanassa in Reverse................. 268

MORGAN, T. H.
The Separation of the Egg of Ilyanassa into Two Parts by Centrifuging.. 280

MORGAN, T. H.
The Rhythmic Changes in Form of the Isolated Antipolar Lobe of Ilyanassa.. 296

BISSONNETTE, THOMAS HUME
Modification of Mammalian Sexual Cycles. II................ 300

OESTING, R. B., AND W. C. ALLEE
Further Analysis of the Protective Value of Biologically Conditioned Fresh Water for the Marine Turbellarian, Procerodes Wheatlandi. IV. The effect of calcium....................... 314

RUNNSTRÖM, JOHN
On the Influence of Pyocyanine on the Respiration of the Sea Urchin Egg.. 327

Volume LXVIII Number 3

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CONTENTS

Page

DAWSON, ALDEN B.
The Hemopoietic Response in the Catfish, Ameiurus nebulosus, to Chronic Lead Poisoning 335

KORR, IRVIN M.
The Relation between Cell Integrity and Bacterial Luminescence 347

EVANS, LLEWELLYN THOMAS
The Effects of Antuitrin S and Sheep Pituitary Extract on the Female Lizard, Anolis carolinensis 355

MENKE, JOHN F.
The Hemolytic Action of Photofluorescein 360

SHAPIRO, HERBERT
The Validity of the Centrifuge Method for Estimating Aggregate Cell Volume in Suspensions of the Egg of the Sea-Urchin, Arbacia punctulata 363

RUNNSTROM, JOHN
An Analysis of the Action of Lithium on Sea Urchin Development 378

WISLOCKI, GEORGE B.
The Lungs of the Manatee (Trichechus latirostris) Compared with Those of Other Aquatic Mammals 385

JOHNSON, MARTIN W.
The Developmental Stages of Labidocera 397

DROUET, FRANCIS, AND AARON COHEN
The Morphology of Gonyostomum semen from Woods Hole, Massachusetts 422

LOEB, LEO
Comparison of the Reactions Against Heterotransplanted Tissues in Different Kinds of Hosts 440

TYLER, ALBERT
On the Energetics of Differentiation. II. A Comparison of the Rates of Development of Giant and of Normal Sea-Urchin Embryos 451

VOLUME INDEX 461

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